A Functional Dissection of Abnormal Signal Processing Performed by the Somatosensory Cortex of young \textit{Fmr1-KO} mice

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Declaration

I hereby certify that this thesis and its composition are entirely my own work, with the exception of example immunohistochemistry shown on P53, assisted by Xiao-qing Yuan in the Laboratory of Chris McBain at NICHD. No part of the work contained in this thesis has been submitted for any other degree or professional qualification.

Signed (candidate)

Date 9/4/2014
Abstract

Every second throughout life, cortical circuitry efficiently compresses and interprets huge volumes of incoming sensory information. This high fidelity sensory processing guides normal brain development and is essential for animals’ successful interaction with the environment. Low-level sensory perceptual disturbance is nearly ubiquitous in Autism Spectrum Disorder (ASD), but despite the potential to offer crucial insight into the abnormal development of higher brain function is poorly understood. Fragile X Syndrome (FXS) is the most common heritable cause of ASD. Previous studies in the Fmr1-KO mouse model of FXS report cell-intrinsic, synaptic and local connectivity abnormalities in the neuronal physiology of primary sensory cortices. This suggests that sensory perceptual dysfunction could emerge from interacting circuit-wide pathophysiology to impair neural adaptations that support high fidelity sensory information processing. However, there is little mechanistic consensus about how this might occur.

To address this, in this thesis I use brain slice electrophysiology and computer modelling to provide a bottom-up description of how thalamocortical (TC) responses, the principal cortical input for ascending sensory information, are mis-interpreted in the somatosensory Layer 4 (L4) circuit in Fmr1-KOs at a crucial developmental transition to active sensory processing. Recruitment of intracortical L4 network activity could be atypically evoked by lower frequency thalamic stimulation in Fmr1-KO slices. Furthermore, profound alterations to single-cell and network response dynamics were observed, in particular loss of spike timing precision considered critical for sensory circuit performance. These network phenomena were supported by interacting single-cell and local circuitry pathophysiology, including hyperexcitable cortical neurons and temporally distorted feed forward and feedback inhibition.

Together, these data demonstrate cortical hypersensitivity to TC inputs and abnormal recruitment of network activity in critical period Fmr1-KO somatosensory cortical circuits. The hyperresponsiveness of intracortical circuitry may underlie tactile hyperexcitability and distorted sensory perception in FXS patients. Interestingly, modelling suggests that many of the alterations of synaptic and neuronal function are compensatory, thus minimizing the impact of the genetic lesion. Thus, this study shows for the first time that circuit level dysfunction emerges in the Fmr1-KO mouse from an accumulation of effects at the synaptic and cellular level; however, it also highlights the challenge of understanding which of these changes are pathological and which are compensatory.
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<th>Description</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
<td>MP</td>
<td>matching pursuit spectral decomposition</td>
</tr>
<tr>
<td>AHP</td>
<td>after-hyperpolarization</td>
<td>MUA</td>
<td>multi-unit activity</td>
</tr>
<tr>
<td>AMPA(R)</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)</td>
<td>NKCC1</td>
<td>Na-K-Cl cotransporter</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
<td>NMDAR</td>
<td>N-methyl-D-aspartate (receptor)</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
<td>P</td>
<td>postnatal</td>
</tr>
<tr>
<td>BAPTA</td>
<td>(1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)</td>
<td>PV</td>
<td>parvalbumin</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotropic factor</td>
<td>RC</td>
<td>resistor-capacitor network</td>
</tr>
<tr>
<td>BK</td>
<td>big Potassium channel</td>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium/calmodulin kinase II</td>
<td>cIIE/IPSC</td>
<td>(spontaneous) excitatory/inhibitory postsynaptic current</td>
</tr>
<tr>
<td>CSD</td>
<td>current-source density</td>
<td>SOM</td>
<td>somatostatin</td>
</tr>
<tr>
<td>CT</td>
<td>cortico-thalamic</td>
<td>STP/D</td>
<td>short-term plasticity/depression</td>
</tr>
<tr>
<td>DC</td>
<td>direct current</td>
<td>STDP</td>
<td>spike-timing dependent plasticity</td>
</tr>
<tr>
<td>DIFC</td>
<td>differential interference contrast</td>
<td>TASK</td>
<td>Tandem pore domain potassium channel</td>
</tr>
<tr>
<td>E/IPS</td>
<td>excitatory/inhibitory postsynaptic potential</td>
<td>TC</td>
<td>thalamocortical</td>
</tr>
<tr>
<td>Ex</td>
<td>excitatory neuron</td>
<td>TRn/RThn</td>
<td>thalamic reticular nucleus</td>
</tr>
<tr>
<td>FFI</td>
<td>feed-forward inhibition</td>
<td>VB</td>
<td>ventrobasal thalamus</td>
</tr>
<tr>
<td>FFT</td>
<td>fast Fourier transform</td>
<td>VGKC</td>
<td>voltage-gate potassium channel</td>
</tr>
<tr>
<td>FMRF</td>
<td>Fragile X mental retardation protein</td>
<td>VPM</td>
<td>ventral posteromedial thalamus</td>
</tr>
<tr>
<td>FS</td>
<td>fast-spiking interneuron</td>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>G/A</td>
<td>GABA/AMPA ratio</td>
<td>ZAP</td>
<td>impedance amplitude profile</td>
</tr>
<tr>
<td>GABA(R)</td>
<td>(\gamma)-aminobutyric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization activated cyclic nucleotide channel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IEI</td>
<td>inter-event interval</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISI</td>
<td>inter-stimulus interval</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCC2</td>
<td>potassium-chloride symporter 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
<td></td>
<td></td>
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<tr>
<td>L4</td>
<td>cortical layer 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFP</td>
<td>local field potential</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTP/D</td>
<td>long-term potentiation/depression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase cascade</td>
<td></td>
<td></td>
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<tr>
<td>MEA</td>
<td>multi-electrode array</td>
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1. Introduction

1.1. Background

Sniffing, licking, touching, whisking, saccadic vision, auditory fixation, electrolocation and echolocation: perception is an active process involving skilled manipulation of specialised sensors. Animals use these sensors in order to glean accurate information from their environment and inform choices about how to interact with the world to their advantage. Successful animals have sensors adapted to their ecological niche to provide the richest possible gamut of sensory information about their environment: the finer the detail provided by sensory input, the more subtle the change in the environment that can be detected, and the better the choice of reaction that can be formulated. During this internal calculus, up-to-date information from sensors is evaluated against internal knowledge of past sensory experiences. Animals calibrate and learn to manipulate and interpret their sensors developmentally from sensory experience, a process that involves anatomical and physiological plasticity in the brain during early postnatal critical periods – transient periods of heightened sensitivity to altered quality of sensory inputs.

In mammals, disrupted sensory experience profoundly influences the functional development of circuitry in the cerebral cortex. Qualitatively altered or restricted sensory input during critical periods produce lasting alterations to sensory perception and performance impairment during psychophysical tasks (Maurer et al., 2005). While such behavioural anomalies include disruptions in higher cortical areas, dysfunctions in
primary sensory circuits have been classically studied for simplicity, and in critical period experimental animals, the same sensory manipulations lead to circuit-wide changes in stimulus-related physiological activation of primary cortical circuitry (Sengpiel et al., 1999).

In human development, early postnatal critical periods are best described for the visual system: visual acuity in humans increases fivefold during the first six months of life (Maurer and Lewis, 2001), a period of heightened sensitivity to cataracts, strabismus and amblyopia (Lewis et al., 1995). Left untreated, critical period cataracts lead to lasting visual impairment: In addition to altered basic psychophysical performance (typically measured using optokinetic response and grating acuity) at the level of primary visual cortex, disrupted higher visual cortical processing is observed. Recognition of both facial pattern (orienting towards basic geometric “face shapes”) (Salapatek, 1975) as well as emotional valence and familiarity detection (Geldart et al., 2002) are hampered in cataractal neonates.

Such hierarchical disruption suggests that distortions in the output of “primary” cortical areas are sequentially mis-informative during the development of higher-order cortical circuitry. A parallel can be found in the development of the auditory system: early exposure to a wide tonal range supports precocious language proficiency and perception of syllables/phonemes with a compressed (more adult-like) tonal dynamic (Kuhl, 2004).

Furthermore, normal social contact – conveying a battery of cognitive, linguistic and sensory stimuli - is important during early development:
impoverishment of such stimulation has profound negative sequelae: Retarded cognitive trajectories are observed both in monkeys subjected to total social isolation during early postnatal development (Harlow et al., 1965) and human infants raised in poor standards of institutional care (Chugani et al., 2001; Nelson et al., 2007; Fox et al., 2010), placing emphasis on beneficial early intervention in both cases. Among the negative cognitive consequences associated with institutional upbringing and social deprivation: self-stimulatory and motor stereotypy, attentional weakness, poor verbal and visual memory, and impulsivity and poor concentration. Furthermore, “sensory oddness” is noted: “preference to be wrapped tightly for sleeping, persistently mouthing objects, bothered by noises” (Chugani et al., 2001; Eluvathingal et al., 2006). In humans, these studies and others demonstrate functional and structural brain abnormalities. Orbitofrontal, prefrontal, medial temporal (amygdala and hippocampus) and brainstem regions show reduced metabolic activity (PET imaging, 18FDG uptake) in children (mean age 8.8 years) reared in institutional care (Chugani et al., 2001). White matter integrity (diffusion tensor MRI) of fibre tracts connecting these regions is reduced in a comparable sample of children (Eluvathingal et al., 2006), suggesting a profound anatomical effect.

The striking overlap between cognitive symptoms of socially restricted children and those observed in children on the autistic spectrum has led to hypotheses that seek to explain abnormal social developmental and cognitive trajectories in autistic children within the framework of altered experience-dependent brain development (Baron-cohen and Belmonte, 2005). Disrupted sensory processing is commonly (nearly ubiquitously)
reported in autistic children and adults (Cascio, 2010). A consequence of qualitatively altered sensory processing or restricted dynamic range of primary cortical circuits during early postnatal critical periods could be to warp the instructive content of these outputs during their development influence on higher-order brain circuitry.

1.3. Organisation of this thesis

What does this mean for neocortical processing in Autism? This thesis seeks to provide a rigorous physiological framework for the formulation of such hypotheses: The behavioural repertoire of an organism must reflect the performance of its circuit-based computations. Therefore, abnormal sensory behaviour must reflect abnormal circuitry. A logical place to start in unravelling perturbations to the function of neocortical circuitry is in somatosensory cortex Layer 4, an area of the brain that matures comparatively early in postnatal development, has a well characterised functional anatomy and in which computational properties have been intensively studied. The ability to present well-controlled stimuli is also crucial. In this study, using a prominent mammalian model of Autism I provide the first description of abnormal signal processing performed by a developing primary cortical sensory network when provided with naturalistic stimuli. The work focuses on the physiology of neocortical Layer 4 in the somatosensory system, where the first stages of neocortical processing are performed on ascending sensory inputs from the whisker periphery.

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1 Yet these symptoms, well evaluated by clinically administered behavioral tests such as the DISCO or Short Sensory Profile (SSP) are not included in the latest diagnostic criteria for autism spectrum disorders, as laid out by the DSM-V.
2 Congenic mice used in the aforementioned study were from the C57bl/6 background, as in the present study.
3 Strong contributions from olfaction are also noted (Myers, 1984).
4 Typified by more motor abnormalities but less delayed language acquisition compared to high-functioning Autistic children, despite a similar age of onset.
5 30% will experience at least two seizures by puberty (Volkmar and Nelson, 1990)
This work describes the physiological state of Layer 4 from the perspective of single cells, local networks and in the larger context of cortical network processing. Experiments reported in the results chapters focus intensely on a crucial two-day developmental time point in the life of *Fmr1-KO* mice - Postnatal (P) days 10-11 - at which Layer 4 is on the cusp of receiving sensory input arising from active whisker exploration. The results of this work provide new insight into potential causative factors in the hierarchically aberrant development that has been demonstrated between cortical laminae, and suggests potential avenues for therapeutic intervention in FXS and Autism.

1.4. Central question addressed by this study

**How is normal Layer 4 circuit recruitment by TC inputs compromised in critical period *Fmr1-KOs***?

As will be discussed below, FS-mediated feedforward inhibition (FFI) in Layer 4 controls the window for synaptic integration in Ex. neurons, such that only highly behaviourally relevant stimuli, (i.e. salient whisker inputs), can activate the recurrent excitatory circuitry, and evoke activity that propagates to the next stage of processing in LII/III. As the principal recipient layer of thalamic input to the cortex, Layer 4 effectively “gates” ascending sensory input to the cortex through precisely timed FFI circuitry. Physiological factors important in the development of FFI have been elucidated by the Isaac lab and collectively support an increase in the temporal selectivity for high-frequency synaptic TC input by Layer 4, a necessary step in the maturation of sensory information processing. As
discussed above, contributions from cell-intrinsic properties, synaptic connectivity and synaptic kinetics are all key factors in this process – pathophysiological changes in all of which have been implicated in FXS. How do these effects synergise in the developing layer 4 to disrupt sensory gating in the circuit?

Previous studies strongly implicate FMRP in normal GABAergic development (Gibson et al., 2008; Olmos-Serrano et al., 2010; Paluszkiewicz et al., 2011). Since absence of FMRP during this developmental period disrupts maturation of the thalamocortical input to layer 4 (Harlow et al., 2010), it is plausible that the emergence of FFI could be similarly compromised in *Fmr1-KOs*, particularly considering that FMRP is expressed in PV<sup>+</sup> FS interneurons (Selby et al., 2007; Till et al., 2012), the strongest source of FFI in layer 4 (Sun et al., 2006).

It will also be important to verify that development of internal chloride ion homeostasis occurs normally: In the *Fmr1-KO* mouse, one study reporting unaltered KCC2 and NKCC1 expression in tissue from whole-forebrain dissections at P5, P12 or from adults. However expression levels were altered for other protein machinery involved in GABAergic transmission including GABA<sub>A</sub> receptor protein subunits and the enzymes GABA transaminase, succinic semialdehyde dehydrogenase and GAD65 (Adusei et al., 2010). This would suggest that despite predicted changes to levels of GABA synthesis, synaptic clearance and degradation, no alterations in

---

2 Congenic mice used in the aforementioned study were from the C57Bl/6 background, as in the present study.
internal [Cl\textsuperscript{-}] would be expected in recordings from these mice. However, this study did not verify this with physiological recordings.

GABAergic integration has been proposed to play a dominant role in the closure of the critical period for synaptic plasticity in sensory cortices (for review see Hensch, 2005). Given the previously described defects in plasticity mechanisms that occur in models of FXS, it is conceivable that in the 	extit{Fmr1-KO} mouse an early deficit in inhibitory Layer 4 circuitry, potentially including altered feed-forward inhibitory function, could contribute to the delayed critical period plasticity recently observed by Harlow and co-workers (Harlow et al., 2010). Furthermore, it is proposed that this could result from a combination of both synaptic and cell morphological components - which in the normal animal develop in tandem during the highly critical P6-P8 period – thus rendering local inhibitory circuits inefficient early in the second postnatal week, at the start of the LII/III critical period. This would complement the observed defects in this ascending connection (Bureau et al., 2008), and feedback inhibitory circuit defects in the adult as demonstrated by circuit hyperexcitability (Gibson et al., 2008). Overall, defects in the maturation of inhibition in layer 4 circuitry and by extension, the maturation of timing precision and sensory processing, are predicted to contribute to abnormal downstream processing, and the subsequent synaptic plasticity and cortical development thereby controlled.
1.5. Novel contributions of this thesis

Overall, findings from *Fmr1-KOs* develop this theme in three results chapters:

- **Chapter 3** provides a detailed characterization of the single-cell electrophysiology of two major classes of neuron in somatosensory cortical Layer 4, as well as their synaptic interconnectivity and a broad analysis of their synaptic inputs. Work in this chapter demonstrates that seemingly small changes to intrinsic excitability have far-reaching consequences for the spectrotemporal representation of synaptic information.

- **Chapter 4** investigates the representation of thalamocortical inputs in the subthreshold and firing responses of Layer 4 excitatory neurons, with particular focus on the efficacy of local feed-forward inhibition. Through computational reconstruction of dynamic thalamocortical frequency sensitivity of Layer 4 from physiological data, I further examine the contributions of individual circuit phenotypes to the emergent behaviour of the circuit and predict an emergent circuit-level dysfunction. This prediction is explicitly verified with electrophysiological recordings.

- **Chapter 5** explores the representation of thalamocortical responses by recurrent network activity in Layer 4. The firing patterns of single neurons, and their synaptic inputs from network feedback are characterized and show profound disruptions in timing and fidelity. A neuronal population level network view is provided by multi-electrode array recordings of widespread cortical activity, which shows widespread distortions to the spatiotemporal propagation of network activity.

Throughout, comparisons are made to the normal developmental progression of cortical development, and to previous work on sensory...
deprived development. The consequences of a lower fidelity, misinstructive sensory representation in Layer 4 for the sequential development of cortical circuits are discussed.

1.6. Literature review

This introduction and literature review is in three parts:

• Firstly, after briefly reviewing the importance of sensory input in normal development, I review sensory abnormalities in autistic patients, and discuss the potential contribution to aberrant cognitive development. I discuss the large etiologic and syndromic diversity of Autism and identify Fragile X Syndrome as a useful model framework for the study of intellectual disability and Autism. I then examine the genetic and biochemical basis of Fragile X Syndrome and survey current literature on physiological development and circuit dysfunction, focusing on mouse genetic models.

• Secondly, in order to justify the choice of model system for this study, I provide background into the modular columnar architecture of cortical networks, and the functional physiology of neurons that connect to form these cortical circuits, with particular reference to primary sensory cortex. I discuss critical developmental phases of single neuron and network physiology that provide milestones in circuit maturation and in animal behaviour. I identify the interpretation of patterns of thalamocortical signals by neocortical Layer 4 as a crucial first step in the cortical reconstruction of the sensory world. I then examine motifs of microcircuit physiology that provide advantageous computational
analogues to the necessary steps in processing of naturalistic sensory stimuli.

• Finally, I hypothesize that changes in the processing of sensory input common in autistic patients is a consequence of defects at the first level of cortical signal processing, the interpretation of thalamocortical input by cortical layer 4, and could arise from multiple interacting circuit phenotypes.

1.6.1. The primacy of touch

Of all sensory modalities, the early evolutionary specialisation of somatosensation is considered to have had the greatest contribution to the early success of the mammalian lineage (Kaas, 1989; Butler, 2008; Rowe et al., 2011). Social interaction between early hominids was likely dominated by tactile behaviours such as grooming (Hertenstein et al., 2006). Later, only as group size and territorial range expanded did the need for distal sensory modalities overtake that of touch for communication, providing selective pressure for vocalisations that led to vocal communication. Consequently, tactile sensation has been identified as a precursor to verbal communication (Dunbar, 1996).

The phylogenetic primacy of somatosensation is mirrored by its rapid developmental acquisition: somatosensory responses are detectable in the human foetal brain at eight weeks post-conception (Montagu, 1987). These develop during gestation (median nerve stimulation in pre-term and full-term were compared by MEG in Pihko and Lauronen, 2004), and while

---

3 Strong contributions from olfaction are also noted (Myers, 1984).
immature at birth (Lauronen et al., 2006) are employed by neonates in the primitive reflexes; including rooting, sucking and palmar grasping. Moreover, sensorimotor integration depends first on touch for calibration, only later with input from vision (Corbetta and Snapp-Childs, 2009).

In addition to the well-recognised role of touch in gross and fine motor skill development (Case-Smith, 1995), reciprocal mother-infant touch provides reassurance and comfort, and builds attachment (Myers, 1984; Jean and Stack, 2009), forming a template for subsequent interaction with family and peers.

1.6.2. Ubiquity of disruption to sensory processing in Autistic Spectrum Disorders

The widespread variability between individuals diagnosed with Autistic Spectrum Disorder (ASD) and associated neurodevelopmental disorders is an intriguing hallmark of the conditions. The severity of intellectual disability (ID) and cognitive impairment, developmental trajectory and severity of epilepsy are all highly variable between individual cases. Strikingly however, atypical sensory symptoms, or behavioural responses to sensory information, are common to individuals across this spectrum (notably reviewed recently in Cascio, 2010; Marco et al., 2011). Furthermore, the overwhelming majority of Autistic children and adults show sensory hyper/hyposensitivities in multiple modalities, ranging in severity. This can be distressing for patients and lead to self-injurious behaviour, as well as inducing anxiety and withdrawal away from normal social situations, as explored in Figure 1-1.
Roles for sensory processing deficits in ASD developmental trajectories: relationship to primary neurophysiological findings

**Auditory**
- Show aversive, hypersensitive behavioral responses to harmless noises
- Altered auditory filtering (Leekam et al. 2007)
- Altered Latency/amplitude of early cortical Event-Related Potentials (EEG, Oram Cardy et al. 2008)

**Impaired language acquisition?**

**Tactile**
- Avoid light touch, clothing and grooming
- Lower amplitude threshold for detection of high frequency vibrotactile stimuli (Blakemore et al. 2006)
- Enhanced early cortical Event-Related Potentials (EEG, Miyazaki et al. 2007)
- Disrupted somatotopic mapping of faces and hands (MEG, Coskun et al. 2009)

**Affected social development?**

**Visual**
- Either seek/avoid visual stimuli, perform poorly on psychomotor tasks
- Impaired dorsal visual stream visual processing (motion processing) (Kogan et al. 2004a)
- Anatomical changes to visual thalamus specifically affecting dorsal stream inputs (fMRI, Kogan et al. 2004b)

**Affected face/emotion recognition, eye contact?**
1.6.2.1. Sensory disruption is lifelong in ASD and spans all sensory modalities

While disrupted multisensory integration has longer been recognised in Autism (O’Neill and Jones, 1997), research has widened more recently to include abnormal unimodal sensory perception. A greater body of work exists for disrupted proximal senses (kinaesthetic and vestibular sensation, touch, smell and taste), considered to represent an earlier developmental abnormality (e.g. Ayres and Tickle, 1980; Baranek et al., 1997), with a greater interest growing for abnormal sensation via distal senses (vision and hearing).

In an early study, Wing (1969) reported that ASD children differed from those with typical development or Down’s syndrome in their odd reactions to sound and visual stimuli, and responses to proximal stimuli. Interestingly, Wing further suggested a parallel to sensory, motor and perceptual problems of deaf-blind children.

More recently, Leekam et al. (2007) noted that current diagnostics (e.g. the Revised Autism Diagnostic Interview, “ADI-R”) collect limited info about sensory responding, and opted instead for the "Diagnostic Interview for Social and Communication Disorders (DISCO)". This test collects detailed sensory information covering a wide range of stimuli, using 21 items related to sensory abnormality that are separated into three groups: proximal, auditory and visual. Other items relating to atypical taste/oral, kinaesthetic, and touch responsiveness are also found in other sections of the DISCO. With this approach, the authors reported that over 90% of ASD individuals (200 tested, aged 32 months to 38 years) had sensory
abnormalities, across multiple modalities. Crucially, proximal domains (touch, smell/taste) that distinguished ASD and control children were broadly unaffected by age or IQ (even in highest functioning individuals). Of the distal sensory domains studied, auditory symptoms remained largely unaffected by age and IQ, whereas visual symptoms showed the greatest sensitivity to these factors and showed slight improvement with age. Taken together, this study showed that younger low-functioning ASD patients were affected more broadly across sensory domains than the other groups. Leekam et al. (2007) also specifically examined a subset of ASD children and reported an increased frequency of sensory symptoms, and greater span of affected sensory modalities in the high functioning autism group compared with the matched comparison group: Over 35% of high-functioning ASD children had abnormalities in nearly all proximal sensory categories. Interestingly, acute hearing could be found in typical children but unusual acuity for particular sounds (e.g. for keys or crisp wrappers) was more prevalent in ASD children.

In adults, two notable studies have provided insight into the lifetime prevalence of sensory disruption. Crane et al. (2009) used self-reporting questionnaires to compare control and ASD cohorts, with the latter mainly high functioning, with many co-diagnosed with Asperger. 94.4% of the ASD subjects reported sensory anomalies - overall they were more sensitive to sensory stimulation and more likely to avoid sensory input. They also registered sensory information less and engaged in fewer sensation seeking behaviours. Interestingly, strong within-group variability was observed for abnormal sensory experiences reported by ASD subjects.
despite similarly severe abnormalities. This suggests that these individuals experience a diversity of sensory hypo/hypersensitivities, in agreement with previously reported disparities. The results of this study are further consistent with autobiographical accounts highlighting the impact of sensory phenotypes in everyday life (Hacking, 2009; Grandin, 2010).

1.6.2.2. Sensory disruptions are distinct between different forms of intellectual disability

Sensory system disruptions largely overlap between ASD children and those affected by other clinical groups with cognitive impairment. Asperger children\(^4\) in particular show strong abnormalities in auditory processing that typically predicts the severity of their distractibility and inattention (Dunn et al., 2002; Myles et al., 2004). Interestingly this recapitulates earlier findings that sensory disruption is greater in high than for low functioning ASD individuals (Freeman et al., 1981).

A similar comparison has recently been made between ASD and FXS patients. Rogers et al. (2003) used a battery of observational tests, including the ADI and Short Sensory Profile (SSP) to score sensory behaviours of toddlers (2-3 years old). Both ASD and FXS patients had more sensory symptoms compared to developmentally delayed and typically developing groups, with differences emerging in specific domains of tactile sensitivity, taste and smell sensitivity and auditory filtering but not for auditory or visual sensitivity. ASD and FXS children were similar in their sensory

\(^4\)typified by more motor abnormalities but less delayed language acquisition compared to high-functioning Autistic children, despite a similar age of onset.
responsiveness, but the ASD group had more extreme scores for taste/smell sensitivity in particular.

Unlike for ASD toddlers, Rogers et al. (2003) found an association between social communication behavior and sensory symptoms in FXS children, who typically showed a poor to moderate communication scale. Interestingly, this trend appears to extend into the general UK population: Robertson and Simmons (2012) recently reported a strong positive association between expression of Autistic traits and the frequency of sensory disruptions, using a voluntary participation online questionnaire. This trend in “neurotypical” participants’ responses should be explored further, and raises intriguing questions about contributions from individual genetic and environmental factors and personal history.

1.6.3. Somatosensory disruption in ASD and FXS

1.6.3.1. Psychophysical studies

A common but overlooked feature of ASD that forms the motivation for this thesis is a hypersensitivity to innocuous tactile stimuli and hyper-reactive aversive response, often manifesting as discomfort with clothes and persistent rubbing following light touch. This “tactile defensiveness” is displayed in individuals with FXS to a greater degree than in typically developing or ASD children (Rogers et al., 2003; Tomchek and Dunn, 2007; Baranek et al., 2008), and has been linked to rigid and stereotyped behaviours, as well as idiosyncratic eating (Baranek et al., 1997).
One hypothesis prompted by these observations holds that perception is heightened in ASD individuals (reviewed in Mottron et al., 2006). Psychophysical evidence suggests this may arise though lower detection thresholds rather than enhanced discriminability: Blakemore et al. (2006) reported a lower perceptual threshold for vibrotactile fingertip stimuli in Asperger children (although see Güçlü et al., 2007), interestingly this was restricted to 200Hz vibrations and not observed for lower frequency (30Hz) stimulation.

A more recent comprehensive study extended this to examine responses to forearm stimulation (Cascio et al., 2008). Interestingly, in ASD children, the authors find a heightened sensitivity to aversive thermal sensitivity, but normal perception of innocuous heat. These effects were specific to the forearm and not observed on the palmar surface. These results are intriguing since the forearm is richly innervated by “C-touch” afferent sensory endings, known to be involved in conveying affective touch (Liu et al., 2007; Löken et al., 2009; Vrontou et al., 2013), and thus may provide insight into a specific abnormality in processing affective touch.

Altered responses during accommodating stimuli have been reported for ASD. Tommerdahl et al. (2007) tested vibrotactile discrimination preceded by an accommodating 25Hz “flutter stimulus” which enhanced tactile localisation in healthy controls, but was less efficient in ASD adults, despite showing a better sensitivity at baseline. The authors suggest that this effect might arise through deficient recruitment of cortical surround inhibition during repetitive stimulation in ASD cases, and that the lateral extent of a cortical minicolumn might be compromised.
1.6.3.2. Neurophysiological and functional imaging studies

Increasingly, abnormal neurophysiological correlates have been reported in tandem with altered behavioural and psychophysical performance (reviewed broadly for ASD by Marco et al., (2011), and in detail for FXS in Knoth and Lippé, 2012). Typically, these studies examine evoked neural activity using functional Magnetic Resonance Imaging (fMRI) and electro- or magneto-encephalography (EEG and MEG, respectively).

A pressing goal is to determine whether disrupted sensory-evoked responses emerge from sensory peripheral, thalamocortical, or intracortical components of sensory processing. Extensive work has been performed in the auditory system, motivated by language deficits observed in ASD. Two notable EEG studies reported slower latencies for early tone-evoked potential latencies in auditory brainstem (Rosenhall et al., 2003; Kwon et al., 2007), however, more complex sounds or speech typically reveal more disrupted late potential components for ASD and Asperger patients (Russo et al., 2008, 2009; Källstrand et al., 2010). In the auditory cortex, disruptions reported by MEG and EEG are divergent: with both earlier (Martineau et al., 1984; Ferri et al., 2003) and delayed (Bruneau et al., 2003; Oram Cardy et al., 2008; Whitehouse and Bishop, 2008; Roberts et al., 2010) early tone-evoked potentials reported. Changes in response latency to more complex sounds, including vowels have also been reported (Whitehouse and Bishop, 2008). The authors of this study also suggest that top-down modulation of primary auditory processing may also be affected.
In primary somatosensory cortex, short-latency event-related potentials following median nerve stimulation were reported enhanced in Autistic compared to neurotypical children, and additionally displayed strong hemispheric disparity (Miyazaki et al., 2007). Whether these low-level differences are a peripheral or short-latency intracortical effect has yet to be revealed, however the short latencies involved implicate early (possibly thalamocortical) transmission rather than complex cortico-cortical interactions. Nonetheless, additional reports implicate disrupted functional somatotopic segregation in somatosensory cortex between tactile input to hand and face (Coskun et al., 2009), and between adjacent digits (Coskun et al., 2013). This has further raised the possibility of altered intracortical surround inhibition and functional columnar size.

1.6.3.3. Functional disruptions to thalamocortical system in ASD and FXS

Input from the thalamocortical system plays a crucial role in early anatomical (Schlaggar and O’Leary, 1991) and functional (Minlebaev et al., 2011) neocortical development. However, the disruption of thalamocortical functional development portrayed by the above ASD studies presents an incomplete picture.

Functional magnetic imaging in ASD adults shows enhanced thalamocortical, but weaker intracortical functional connectivity, albeit using slow BOLD signal correlation (Mizuno et al., 2006). One possibility is that this arises through enhanced thalamocortical transmission. Belmonte et al. (2004) have suggested that altered functional connectivity may arise
in Autism from disrupted arousal modulatory systems. In particular, this is crucial for control of the thalamocortical system, where (notably) cholinergic modulation of T-type currents in thalamic relay neurons switches them between tonic and burst firing modes (reviewed in Sherman and Guillery, 2002), accounting for transitions in thalamocortical oscillations observed between sleep and arousal (Steriade et al., 1993).

Direct evidence implicates defective thalamocortical gating in ASD. It has long been known that short-latency auditory-evoked cortical potentials are significantly enhanced in ASD compared to control children during REM sleep (Ornitz et al., 1968, 1973). This enhanced arousal likely contributes to the poorly consolidated sleep typical in ASD children, although interestingly cortisol levels are not enhanced, distinguishing the condition from clinical insomnia (Limoges et al., 2005).

In addition to affecting thalamic gating, altered thalamocortical transmission could further distort the fidelity of information transmission from the sensory periphery to the cortex. In particular, thalamocortical transmission is associated with a transformation between a time-based thalamic code to a cortical rate code in the somatosensory system (Ahissar et al., 2000). Notably, thalamocortical responses by neurons in cortical layer 4, the principle destination for lemniscal thalamocortical axon terminals, provide a frequency-dependent gate to ascending sensory information (Beierlein et al., 2002).

Strikingly, a recent functional imaging study (Dinstein et al., 2012) reported lower trial-to-trial fidelity of sensory evoked responses in primary sensory
cortices of high-functioning ASD adults – spanning auditory, visual and somatosensory modalities. These differences were not observed in thalamic nuclei, and did not involve altered resting state activity (although default mode abnormalities have been reported – reviewed in Broyd et al., 2009). The authors previously reported reduced auditory-evoked interhemispheric BOLD synchronisation in the superior temporal and inferior frontal gyri of sleeping Autistic toddlers (Dinstein et al., 2011). These areas are involved in communication and the extent of the disruption correlated well to individual language impairment scores. Taken together, the authors interpret the findings of these two studies as relatively unaffected thalamocortical pathways, but strong intracortical disruption.

Most recently, in a study of ASD children aged 9-19, Nair et al. (2013) reported both anatomical and functional hypoconnectivity between five pan-cortical areas and thalamic nuclei, but acknowledged that the direction of reduced connectivity remains unverified. When compared to the older aged cohorts of Mizuno et al. (2006) and Dinstein et al. (2012), it is possible that protracted developmental mechanisms could be involved in the transition between the observed thalamocortical phenotypes.

Overall therefore, these results present a mixed picture of interacting thalamocortical and cortico-cortical abnormalities in ASD and highlight three main points: the difficulties inherent in testing developing Autistic children, the widespread consequences of early sensory disruption, and the need for a more detailed understanding of the neurophysiological basis for disrupted sensory processing in neurodevelopmental disorders.
Due to the widespread nature of the above disruptions to cortical processing, attention will now turn to functional cortical development in ASD. Similarly, functional and anatomical development of sensory thalamus is almost certainly disrupted; in particular, the cellular anatomy of the lateral geniculate nucleus has been implicated in disrupted dorsal stream vision in FXS (Kogan et al., 2004a, 2004b). However work in this thesis will capitalise on the large body of published work exploring the development of the functional columnar architecture.

1.6.4. Functional cortical columns: lateral interactions and sparse firing

In order to formulate hypotheses on how cortical processing is affected in ASD is important to now closely examine the structure and function of cortical microcircuits, and identify potential vulnerabilities in the emergent circuit properties supporting information coding. Cortical columns are considered to be the functional unit of neocortical circuit computation (Mountcastle, 1997). Their anatomically and functionally compartmentalised columnar architecture supports efficient processing of external and internal stimuli. Due to the ability to present well controlled sensory or electrical/optogenetic stimulation, the physiology of primary sensory cortex has become an intense and predominant area of study on cortical development and function.

Notably, spiking of cortical neurons is typically sparse, that is, it demonstrates a “Measure of kurtosis or shape of the firing rate distribution” (Rolls and Tovee, 1995) such that cells are generally silent but are highly
stimulus-tuned and fire robustly for a particular stimulus. This has classically been shown for primary sensory cortices, where cells in Layer 4 are biased towards responsiveness to a single eye (Hubel and Wiesel, 1959) or whisker (Welker, 1976), whereas more complex sub- and supra-threshold stimulus specificity in the responses of supragranular layers.

What qualities of neuronal firing are used by cortical circuits to represent sensory inputs? Recordings from sensory cortex identify information transmission in the typically sparse spike patterns both from single neurons and in local populations of cells (Petersen et al., 2001; Okun et al., 2012). Over a fixed set of stimuli, Willmore et al. (2011) make a distinction between “lifetime sparseness” of individual neuron firing rate distributions, and “population sparseness”, reflecting the fraction of an ensemble of cells that fire. Together, these emergent properties help to balance the substantial metabolic costs associated with spike-based communication (Laughlin, 2001), and maximise information transmitted per spike (Levy and Baxter, 1996; Baddeley et al., 1997). In the rodent whisker-responsive (or “barrel”) somatosensory cortex, the definition of sparseness has been expanded to describe neurons or ensembles that have low mean (evoked or spontaneous) spike rates (Brecht and Sakmann, 2002a; Petersen et al., 2002; Panzeri et al., 2003).

In addition to spiking activity within cortical columns, their functional size is also tightly constrained by the spatio-temporal balance between glutamatergic (excitatory) and GABAergic (inhibitory) synaptic activity. Correspondingly, the lateral extent of sensory activation in a cortical column can be modulated by GABAergic blockade (Petersen and Sakmann,
Furthermore, GABAergic inhibition supports centre-surround sensory response profiles that decorrelate firing patterns (Vinje, 2000; Haider et al., 2010), and increase stimulus selectivity and precision (Webber and Stanley, 2006; Jacob et al., 2008). In the visual system, strong surround suppression is ubiquitous, where it increases contrast and provides edge detection, additionally reducing information redundancy in natural scenes by negating responses to constant illumination. In the auditory and olfactory systems, surround suppression has also been implicated in sharpening frequency and odour detection thresholds, respectively.

In the somatosensory system, surround suppression is thought to lower two-point discrimination thresholds for cutaneous stimulation (Laskin and Alden, 1979). Compared to the role of surround inhibition the visual system, consensus lags somewhat in the somatosensory system literature (reviewed in Sachdev et al., 2012), owing in part to the incomplete grasp of the whisker system’s optimal “natural scene”. Also, compared to the visual system, with its dramatically higher receptor count and emergence of columnar responses only at the level of V1, individual whisker functional groups can be found in brainstem and thalamus in the somatosensory pathway. Furthermore, each of stages show multi-whisker responses, albeit slower/weaker for surround whiskers, and thalamic relay neurons show emergent whisker direction sensitivity (Petersen et al., 2008). This suggests an incremental development of centre-surround interactions in the whisker pathway, rather than an abrupt emergence in the cortex, as found in V1 for orientation selectivity from direction sensitive thalamic afferents from the Lateral Geniculate Nucleus (Cruz-Martín et al., 2014).
In the barrel cortex, inhibition and excitation are balanced both at rest and during processing of sensory information (Hasenstaub et al., 2005; Okun and Lampl, 2008), but sensory input can differentially modulate the two synaptic components. Both the the cortical state and the whisker stimulation history influence the amount of inhibition recruited by sensory input (Erchova et al., 2002; Petersen et al., 2003; Sachdev et al., 2004; Hasenstaub et al., 2007; Heiss et al., 2008). This is also reflected in the differential recruitment of membrane potential synchronisation between different classes of inhibitory and excitatory neurons by sensory input (Gentet et al., 2010). Long-range horizontal GABAergic projections spanning multiple columns provide surround inhibition in Layer 2/3 (Adesnik and Scanziani, 2010), while interneurons that project between neighbouring barrels have been reported in Layer 4 (Helmstaedter et al., 2009). Finally, it is important to consider however, that surround suppression can also arise through the withdrawal of excitation (Higley and Contreras, 2003, 2005a), since centre-surround adaptation persists somewhat when the GABA_A agonist muscimol is applied to the cortical surface.

Overall, Sachdev et al. (2012) propose a general rule for sensory systems whereby naturalistic sensory input contains spatially-extensive stimuli that activates both centre and receptive surround fields of most sensory organs. Multi-level lateral interactions then contribute to increasing both the sparseness and selectivity of sensory cortex.
1.6.4.1. Disrupted columnar organisation in the autistic neocortex

Some evidence suggests that the columnar anatomy is abnormal in ASD neocortex. Casanova et al. (2002a, 2002b, 2002c) have reported narrower and more numerous cortical minicolumns in post-mortem tissue from ASD and Asperger patients. Minicolumns typically contain 60-100 excitatory neurons, with 60~80 minicolumns estimated to form a single cortical (macro)column (Favorov and Kelly, 1994a, 1994b). Reelin, a glycoprotein secreted from superficial Cajal-Retzius cells (Nishikawa et al., 2002), has been implicated in abnormal excitatory columnar development in autism (Fatemi, 2002; Fatemi et al., 2005), however focus has turned towards potential disruption of inhibitory circuitry in ASD, due to the observation of wider cell-sparse regions of neuropil surrounding the minicolumn. Casanova and colleagues (2003) have argued that this might represent an anatomical substrate for altered surround inhibition, since these areas are rich in unmyelinated GABAergic projections and axon-dendritic appositions (Buxhoeveden and Casanova, 2002), notably for descending chandelier, double-bouquet and basket interneurons (DeFelipe et al., 1990; DeFelipe, 1999). These superficial interneurons migrate primarily from the caudal ganglionic eminence (Corbin and Butt, 2011) and are postulated to provide both dis-inhibition of within-column Layer 4 interneurons, and inhibition of surrounding columns.
1.6.4.2. Models of columnar disruption in ASD

What are the physiological effects of these columnar In/Ex disruptions? Several modelling studies have investigated the effects of altered surround inhibition on columnar function. Firstly, the classic model of visual cortex functional columnar organisation and feature tuning proposed by Hubel and Wiesel (1962) involves excitatory convergence exclusively, without an inhibitory component. Subsequent models showed that intracortical, rather than feed-forward thalamocortical inhibition could explain cross-orientation suppression in excitatory columnar models (Vidyasagar et al., 1996; Sompolinsky and Shapley, 1997). These provided a consensus view that excitatory interactions broaden functional columns by fusing minicolumns, whilst inhibition acts to subdivide columns into smaller functional units. In order to explore ASD symptoms of stereotypy and selective attention to detail, Gustafsson (1997) implemented a model of cortical map self-organisation using “Mexican Hat” profiles of surround inhibition. He argued that situations with narrower columns favour discrimination while wider ones favour generalisation, relying on a distribution of synaptic weights within the columnar architecture. This builds on an earlier study in which Cohen (1994) used an artificial neural network to argue that excessive connectivity within the hidden layer of “neurons” led to over-fitting of the training set, that is, it showed excessive discrimination but poor generalisation.

The model proposed by Gustafsson (1997) raises some issues: problems include an inability to explain heightened seizure prevalence in ASD
children\(^6\) (which are typified by synchronisation rather than discretisation), and other unaccounted sources of inhibition within the cortical column, such as thalamocortical feed-forward inhibition. Nonetheless, this study does capture some behavioural features reported in ASD, notably enhanced discrimination performance in visual search tasks, considered to represent low-level perceptual abnormalities in primary sensory cortex (Plaisted et al., 1998; O’Riordan and Plaisted, 2001; O’Riordan et al., 2001; O’Riordan, 2004).

1.6.5. Neuronal input-output gain

In the neocortex, tuning of neuronal firing in response to features of synaptic input aids discrimination among qualitatively similar patterns and imparts flexibility to cortical circuit computations. The abstracted, hierarchical organisation of neocortical circuits is proposed to allow successively more nuanced feature extraction from ascending inputs (e.g. Carandini 2012).

The emergent feature selectivity is a truly circuit-distributed property, for example, the orthogonal orientation tuning that can be observed in the spike output of (at least in rodents) side-by-side pyramidal neurons in primary visual cortex is dependent upon the dendritic integration of intermingled synaptic inputs rather than through an ordered, (i.e. dendritic branch-specific) organisation (Jia et al., 2010; Chen et al., 2012). Furthermore, neuronal input-output transformations are cell-type specific (Ma et al., 2010; Runyan et al., 2010; Hofer et al., 2011; Petersen and

\(^6\) 30% will experience at least two seizures by puberty (Volkmar and Nelson, 1990)
Crochet, 2013), which can be used advantageously by the experimenter to objectively discriminate between cell classes from experimental parameters such as intrinsic physiology and anatomy (Ascoli et al., 2008; Brown and Hestrin, 2009). Moreover, both within-class and inter-class response heterogeneity are computationally advantageous; helping to decorrelate responses between cells sharing common synaptic inputs thereby reducing information redundancy among neurons (Padmanabhan and Urban, 2010; Angelo et al., 2012; Litwin-Kumar and Doiron, 2012; Tripathy et al., 2013).

1.6.5.1. Intrinsic neuronal properties regulate input-output gain

The tuning of neuronal firing requires several physiological specializations. In particular, the interaction between synaptic location and intrinsic membrane properties strongly predict the contribution of a given synaptic input towards causing a cell to fire (Komendantov and Ascoli, 2009; Branco and Häusser, 2010; Silver, 2010). Furthermore, synaptic position and dendritic morphology dictate the response of postsynaptic voltage changes and intracellular signalling parameters such as the rate of calcium concentration rise (Jaffe et al., 1992; Vetter et al., 2001), both implicated in the sign and magnitude of synaptic plasticity (Bi and Poo, 2001).

The extent to which impinging dendritic synaptic input will cause deflection in the membrane potential, and by extension, contribute to firing is dictated by the intrinsic excitability of the postsynaptic neuron (reviewed in Silver 2010). The subthreshold current-voltage relationship, as captured experimentally in an I-V plot, is contributed to by a multitude of linear and non-linear membrane conductances (Hodgkin and Huxley, 1952; Koch,
1999) but is dominated by a linear (i.e. Ohmic) contribution by two-pore K+ leak channels (Enyedi and Czirjak, 2010). Thus, the input resistance linearly scales voltage response to current input over an extended subthreshold range. Suprathreshold, the relationship between magnitude of current input and frequency of spiking forms a sigmoidal function that has both a current offset (”rheobase”) and a gain (“F-I” slope, measured in Hz/nA). Both mathematical operations are utilized by neurons to maintain their input-output curve within a useful dynamic range, matching narrow firing ranges of available firing rates to wide ranges of potential input strengths. A subtractive offset shift can account for a level of tonic (baseline) excitatory input, while multiplicative (slope) scaling adjusts the output gain in response to magnitude of input changes. In addition to subtractive modulation of F-I slope through homeostatic regulation of input resistance by ion channel expression (e.g. Turrigiano and Nelson 2000; Destexhe and Marder 2004), properties of natural (i.e. noisy, spectrally-rich) stimuli such as balanced inhibitory/excitatory synaptic input (Chance et al., 2002; Mitchell and Silver, 2003) and short-term plasticity of synaptic input are also important contributing parameters (Abbott et al., 1997; Abbott and Regehr, 2004; Destexhe and Marder, 2004; Cardin et al., 2008; Rothman et al., 2009).

7N.B. shunting of input resistance occurs in vivo as a result of greatly elevated synaptic input rate (e.g. inhibitory input during visual stimulation Borg-Graham et al. 1998) and is known to impose divisive gain control upon spike output.
1.6.5.2. Frequency-dependent filtering effects of neuronal membranes

Neuronal voltage responses following synaptic input are not instantaneous⁸: The intrinsic capacitance of the neuronal lipid bilayer membrane slows the voltage change in response to accumulating synaptic charge by an exponential time constant dictated by the product of the neuron’s membrane resistance and capacitance. In addition to imparting low-pass filtration to the voltage transformation performed upon synaptic input, this effect introduces a significant current-voltage phase lag during responses to fast oscillatory synaptic activity. Consequently, the neuronal input-output transform is a frequency-dependent operation, to the extent that dramatic voltage attenuation at the soma will be observed between two anatomically identical dendritic synaptic inputs are stimulated at different frequencies (Zador et al., 1995; Carnevale et al., 1997).

1.6.5.3. Developmental maturation of neuronal excitability

Input-output transformations by single cells and microcircuits are developmentally regulated by diverse circuit strategies (Daw et al., 2007a; Hull et al., 2009)⁹. Both glutamatergic and GABAergic synaptic connectivity (Shepherd et al., 2003; Daw et al., 2006, 2007a; Chittajallu and Isaac, 2010; Ashby and Isaac, 2011) and neuronal excitability (e.g. Okaty et al. 2009; Sun 2009; Breton and Stuart 2011; Miller et al. 2011) follow developmental

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⁸ Fundamentally limited by the impossibility of passing infinite current. ⁹ In the adult brain, responses are distinct among neighbouring embryonically- and adult-born neurons (Nakashiba et al., 2012).
trajectories that are sensitive to perturbations in synaptic input either through pharmacology or sensory deprivation.

In barrel cortex Layer 4 neurons, the maturation of intrinsic membrane properties during the first postnatal week is a crucial component of overall functional circuit development. In excitatory neurons, maturation of the dendritic arbour through selective retraction and elaboration of dendrites achieves preferential dendritic orientation towards the barrel hollow (Greenough and Chang, 1988). The resulting increase in neuronal membrane surface area causes opposing shifts in membrane resistance and capacitance (Daw et al. 2007, supplementary materials) that differentially affect the membrane time-constant. During the same timeframe, the input resistance of Fast-Spiking GABAergic interneurons drops by half, and firing rate increases in response to injected current. By P10, the input resistance has dropped further and spike waveforms become characteristically narrow, brought about by the up-regulation of TASK and K\(_{3.1}\) channel expression respectively (Okaty et al., 2009). In particular, the presence of Kv3.1 provides characteristic “Fast-spiking” abilities (Du et al., 1996; Rudy and McBain, 2001; Lai and Jan, 2006; Goldberg et al., 2010). A further adaptation in FS cells at this age is the onset of Parvalbumin expression; a calcium buffer that permits sustained FS firing at very high (<100Hz) rates without the cytotoxic consequences of Ca\(^{2+}\) accumulation.
1.6.6. Structure and function of the barrel cortex

1.6.6.1. Ethology and gross anatomy

The rodent somatosensory system has become the prominent model in the study of sensory circuit development and function. It is particularly amenable to the study of neocortical columnar development as the cortical representation of sensory input from the myastacial vibrissae - highly behaviourally salient and strongly represented in rodent cortex - faithfully recapitulates the topology of the whiskers on the snout. Whisker tactile information is conveyed by Ventrobasal thalamic nuclei, which project most strongly to cortical layer IV (Layer 4), where they form excitatory connections with spiny stellate cells, the principal excitatory cells in Layer 4. Stellate cells developmentally segregate to form a cell dense wall and cell sparse hollow, clustering around thalamic inputs from a single whisker and later preferentially orienting their dendrites to converge upon principle whisker thalamocortical axon termini (Fox, 2002). Identifiable under light microscopy, this repeating circular arrangement forms a “barrel” pattern (Woolsey and Van der Loos, 1970) schematised in Figure 1-2a, with each barrel representative of a single cortical column, whose cells show maximal and largely barrel-confined response to input from a principle whisker (Petersen and Sakmann, 2001). Readily discernable columnar anatomy, coupled with the mouse’s amenability to genetic manipulation, make the barrel cortex an excellent system in which to study activity-dependent circuit development.
Figure 1-2 Gross anatomy of the whisker pathway.

a). Somatotopic mapping or architectonic structures between snout and cortex. b). Laminar structure of lemniscal thalamocortical innervation from Ventral posteromedial thalamus (VPM). b). is redrawn from (Feldmeyer et al., 2012)

1.6.6.2. Coincidence detection at thalamocortical inputs to Layer 4

Layer 4 neurons play a key role in the canonical lemniscal sensory pathway by receiving the first cortical input for ascending sensory input via thalamocortical (TC) axons (green in Figure 1-2b), which providing a major source of synaptic input from the periphery. In Layer 4 of the barrel cortex, a highly recurrent (but non-random) network of ~800 excitatory neurons receive lemniscal input from 80~200 thalamic projection neurons via overlapping projections that are both strongly convergent and divergent. Individual thalamocortical inputs to Layer 4 excitatory neurons are weak, providing unitary thalamocortical EPSPs of ~0.5mV in postsynaptic Ex.

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10 This view is currently being challenged however by new evidence for the role of thalamocortical input to deeper cortical layers (Constantinople and Bruno 2013; Wang et al. 2013, M. Daw, personal communication).
neurons; typically unable to drive cortical firing individually (Bruno and Sakmann, 2009). These authors reason that in order to evoke for example, the subthreshold depolarization provided by whisker deflection in the preferred direction (~15mV), the coincident discharge of an estimated 85 TC axons, (or, ~600 dendritic spines) would be necessary. Despite their individually weak connections, TC synapses are highly reliable, showing a probability of synaptic release notably higher than many central synapses (Gil and Amitai, 1996; Stratford et al., 1996; Gil et al., 1999). Taken together, these data have been used to argue that the TC projection is specialized to sense temporal correlations among ascending sensory thalamic inputs, such that only high frequency thalamic firing characteristic of active sensory exploration is capable of evoking postsynaptic voltage summation and driving a postsynaptic spike (Bruno and Sakmann, 2009). In a computational study on the fidelity of information propagation by synchronous TC firing, Wang and colleagues (2010) note that only 20~40 TC inputs, firing within 5ms are sufficient to reliably increase cortical spike fidelity, but that the relative balance or cortical inhibitory and excitatory inputs was a crucial factor.

Strong nonlinear synaptic integration from dendritic NMDA conductances in Layer 4 Ex. neurons plays an important role in information processing (notably, whisker direction sensitivity) in adult barrel cortex (Lavzin et al., 2012). Neonatal TC synapses also possess a prominent Mg$^{2+}$-insensitive NMDA component (Binshtok et al., 2006) with a contribution to TC EPSCs at resting potential that is down-regulated from 40% to 20% between P6 and P11 (Chittajallu and Isaac, 2010). Thus, slow currents provided at
resting potential by this NMDA conductance (and other sources of slow currents, potentially including kainate receptors, e.g. Kidd and Isaac 1999), contribute to synaptic integration over 100s of milliseconds.

1.6.6.3. Thalamocortical feed-forward inhibition

Faithful representation of spike times evoked by sensory input is particularly important for the somatosensory system (Grothe and Klump, 2000; Ahissar and Arieli, 2001). The slow time constants of intrinsic membrane and synaptic responses might seem at odds with this requirement, however an additional contribution from the local circuit further sculpts response kinetics.

Overcoming the problem of slow intrinsic kinetics in the postsynaptic response, precise timing of thalamocortical input integration by Layer 4 Ex. neurons is dynamically controlled (Gabernet et al., 2005) by feed-forward inhibition (FFI) from thalamically co-activated predominantly Parvalbumin (PV^{+ve}) containing, Fast-Spiking (“FS”) Layer 4 interneurons(Sun et al., 2006), shown fluorescently labelled in figure 1-3.
Figure 1-3 GABAergic Interneurons in the barrel cortex at P11.

A). Immunohistochemistry against Parvalbumin (Ai, red) and GAD-67 (GFP, Aii, green) – positive cells in a thalamocortical slice (Aiii: merge). Enlarged area (white box) is 1mm². B). Laminar distribution of interneurons in S1 cortex, enlarged from A). Blue stain is anti-serotonin transporter (SERT), marking TC axonal ramifications in Layer 4 barrel patches.
FFI is strong and robust, and can be recruited by even a single thalamic action potential (Swadlow and Gusev, 2000; Sun et al., 2006; Cruikshank et al., 2007; Hull et al., 2009; Bagnall et al., 2011). Functional maturation of this circuit occurs during the second postnatal week in rodent S1 (Daw et al., 2007a; Chittajallu and Isaac, 2010) and involves GABAergic synapse formation between initially unconnected FS and Ex. neurons, such that by P10, at the onset of active whisker exploration, all excitatory neurons receive FFI (Daw et al., 2007a). Sensory deprivation during this period disrupts functional maturation of FFI, reducing its effect on Ex. cells, as observed as broadened TC-evoked excitatory post-synaptic potentials (EPSPs) (Chittajallu and Isaac, 2010). Additionally, the direction of the transmembrane ionic [Cl\(^-\)] gradient, established by antagonistic actions of NKCC1 and KCC2 chloride transporters is strongly developmentally regulated (reviewed in Blaesse et al. 2009). GABA\(_A\) currents in Layer 4 excitatory neurons are depolarizing embryonically and during early postnatal life (at least in the barrel cortex: Daw et al. 2007), before becoming shunting/hyperpolarizing towards the end of the first postnatal week. Internal [Cl\(^-\)] can vary considerably between neighbouring neurons of different cell classes and age however, (Overstreet-Wadiche et al., 2005; Banke and McBain, 2006), and considerable inter-cell variability is observed during the developmental shift from depolarizing to hyperpolarizing GABAergic response (Daw et al., 2007a). In other brain regions, this shift in equilibrium potential supports a role for GABA as a “primordial excitatory transmitter”, before the developmental emergence of glutamatergic synaptic connectivity (Rivera et al., 1999; Ben-Ari, 2002; Ben-Ari et al., 2004;
Bonifazi et al., 2009; Valeeva et al., 2010). Importantly, the expression and function of KCC2 is sensitive to the history of synaptic activity (Ganguly et al., 2001; Woodin et al., 2003; Lee et al., 2011a; Puskarjov et al., 2012) and changes to GABA_A synaptic efficacy that follow internal [Cl] shifts have previously been implicated in pathological states such as epilepsy and traumatic brain injury (Lüscher and Keller, 2004; Marty and Llano, 2005; Dzhala et al., 2012; Kullmann et al., 2012).

1.6.6.4. Dynamic control of thalamocortical integration

In addition to strength, the timing and synaptic kinetics of FFI contribute to electrically sculpting the postsynaptic voltage response. For example, relative onset timing and decay kinetics of excitatory and inhibitory currents affect the fine temporal balance of inhibitory and excitatory conductances, with feed-forward inhibition implicated in controlling synaptic integration (Higley and Contreras, 2003, 2006; Pouille and Scanziani, 2004; Gabernet et al., 2005; Pouille et al., 2009).

In the thalamocortical pathway, the efficacy of FFI is dynamically regulated (Gabernet et al., 2005; Higley and Contreras, 2006; Cardin et al., 2010), differentially optimizing the circuit’s frequency response for performance during both the detection of initial contact and the discrimination of whisker input scenes during ongoing active exploration (Garabedian et al., 2003; Moore, 2004). Due to history-dependent effects on synaptic release probability at TC inputs and of FFI onto Ex. neurons, repetitive activation of TC fibres at frequencies relevant to intact sensory input (5~100Hz) leads to short-term depression (STD) of synaptic strength. This process is
asymmetric in the FFI circuit; an additional depressing synapse between FS interneurons and Ex. neurons in Layer 4 further compounds depression at TC inputs to both cell types, leading to a frequency sensitive reduction of FFI efficacy during sustained high-frequency TC activation.

A powerful emergent computational benefit of this arrangement is a frequency-dependent, and thus sensory context dependent, adaptation of the gain control provided by FFI, dynamically lowering the cut-off of high-pass filtering performed on TC inputs by Layer 4 Ex. neurons (Moore, 2004; MacLean et al., 2005; Watson et al., 2008). Since the emergent dynamic frequency responses of FFI circuits are exquisitely balanced by the relative rates of excitatory and FF-inhibitory STD (Klyachko and Stevens, 2006; George et al., 2011; Rotman et al., 2011), small perturbations of either of these parameters could be expected to profoundly change the frequency discrimination performance of TC inputs by Layer 4 circuits. Of particular interest, a “diversity of synaptic filters” can be obtained following seemingly subtle changes to the relative strength and kinetics of FFI (explored in an analogous vertebrate system in George et al. 2011).

1.6.6.5. Anatomical development of the barrel cortex Layer 4 excitatory circuit

In the barrel cortex, responsiveness to sensory input is plastic to changes in sensory experience; either through perturbation (Wallace and Fox, 1999) or by in vitro manipulation (Feldman et al., 1998). Typically\(^{11}\), robust changes to the anatomy or electrophysiological response of Layer 4 cells cannot be

\(^{11}\)with the exception of strong “checkerboard” whisker deprivation, reviewed by Fox and Wong, (2005)
induced past the close of a critical period for synaptic plasticity at the end of the first postnatal week. However, early postnatal sensory perturbations can alter the initial dendritic arborisation of Layer IV stellate cells (Lee et al., 2009). The normal profile of dendritic spine development displays a remarkably rapid increase in spine density between postnatal day 9 and 10 (P9-10) resulting in a 200-fold increase in density by P12 (Ashby and Isaac, 2010). Integration of these spines to produce functional connectivity requires sensory experience, as whisker trimming from birth prevents the observed three-fold increase in connection probability between stellate cells in Layer 4 barrel cortex. Sensory activity is again required between P10-30 in the maturational pruning of these initially overproduced spines (Lee et al., 2009).

Of the intracortical excitatory inputs to Layer 4 excitory neurons (~1800 out of ~2800 spines, as estimated by Bruno and Sakmann (2009) from data in White and Rock 1981 and Lübke et al. 2000), the majority of these (~99%) arise predominantly from recurrent intracortical connections made by other neighbouring excitatory neurons located within the same Layer 4 barrel (White and Rock, 1981; Lübke et al., 2000; Petersen and Sakmann, 2000; Lefort et al., 2009; Feldmeyer, 2012). Like TC synapses, synapses formed by these intracortical connections are robust and show high release probability (Feldmeyer et al., 1999; Gil et al., 1999; Lefort et al., 2009; Feldmeyer, 2012). Together, these findings have driven the hypothesis that the Layer 4 network serves as a “hub of intracortical information processing” that coordinates intracortical signal flow between all cortical layers (reviewed by Feldmeyer 2012). This hypothesis is further supported
by the presence of within-column anatomical projections from Layer 4 to all other cortical layers except Layer 1.

1.6.6.6. Development of firing patterns in sensory cortex

Dogma holds that fast cortical processing involving gamma-band synchronisation (oscillations 30~80Hz) develops relatively late in development, appearing postnatally in humans and continuously developing into adulthood (reviewed in Uhlhaas et al. 2010). However, recent work on the functional development of cortical responses to sensory stimulation during the first two postnatal weeks report discrete shifts in the extracellular LFP and single neuron activity. Studies in both the visual (Colonnese et al., 2010) and somatosensory (Minlebaev et al. 2007, 2011; Yang et al., 2012) systems show prominent gamma activity in early postnatal cortex which changes abruptly to coincide with the developmental onset of patterned sensory-evoked TC activity.

“Spindle bursts”, spindle-shaped early gamma oscillatory patterns in the alpha-low gamma range, are the predominant pattern of network activity in neonatal cortex. These arise in primary sensory cortex from phase-locked glutamatergic and basket (GABAergic) synaptic inputs during sensory spontaneous sensory pathway activity, i.e. muscle twitching in the somatosensory system (McVea et al., 2012; Tiriac et al., 2012) and from the afferent relay of spontaneous retinal waves to the visual cortex (Hanganu et al., 2006).

Several distinctions have been observed between these early developmental gamma oscillations, and sensory-related gamma activity.
that appears later in the second postnatal week, which is thought to rely both on increases in supragranular and intercolumnar connectivity and particularly on the maturation of functional basket inhibition and (reviewed in Khazipov et al. 2013). The relative amplitude, latency and gamma spectral power of EPSCs and IPSCs recording during spindle bursts are developmentally modulated, showing a switch in origin from thalamocortical to intracortical generators (Minlebaev et al., 2011). Crucially however, gamma LFP activity is visible in Layer 4 in young animals before the onset of synaptic input to Ex. neurons from FS interneurons.

Neonatal cortical oscillatory responses are also spatially confined both radially and laterally within Layer 4. Unlike those in older tissue, cortical gamma oscillations are relatively unaffected by blockade of cortical GABAergic input (Colonnese et al. 2010, Minlebaev et al. 2011 Fig S10), whereas they are abolished by intrathalamic gabazine injection (Minlebaev et al. 2011 Fig S6).

1.6.6.7. Early involvement of thalamocortical oscillations in Layer 4 network activity

Taken together, these data have been used to argue that early gamma oscillations are generated in the thalamus and projected onto the cortex, whereas activity observed at older ages requires cortical GABAergic connectivity to support an inhibitory gamma oscillator. Overall, it appears that little recurrent cortical processing takes place at early postnatal ages, rather relying on sustained input from thalamocortical oscillations. This
rhythmic cortical input has been suggested to support TC plasticity (Minlebaev et al., 2011), and furthermore to provide a primitive form of vision (Colonnese et al., 2010), with the ascending inputs sustained and amplified by TC oscillations. Presumably this subserves a prototypical role, before the functional maturation of the Layer 4 recurrent circuit at the onset of patterned synaptic activity from active sensory exploration.

Following the developmental onset of cortical responsiveness to light (at ~P8), Colonnese and colleagues (2010) and Yang and colleagues (2012) define two sequential periods of cortical responsiveness to visual stimulation. Between P8-11, complex oscillations at alpha-gamma frequencies were observed, typically terminated by a prominent negative LFP deflection crested by a burst of multiunit activity. These responses were variable in both time-course and appearance and were notably similar to previously described waveforms of immature cortex, including gamma and spindle bursts, and “delta brushes”. Subsequently, at P12, light evoked oscillations disappeared in Layer 4, while strong MUA remained, typically accompanying a single negative visually evoked LFP deflection. This was similar to the activity patterns found in adult brains.

Eyelid separation takes place in rat pups at ~P14, therefore the changes in cortical responsiveness reported by Colonnese and colleagues (2010) took place coincident with the onset of active vision. A similar functional transition in the touch responsiveness of barrel cortex Layer 4 was reported between P7 and P9, also ~2 days preceding the developmental emergence of active whisking (also explored in greater detail in Minlebaev et al. 2011).
Interestingly, these changes coincide with increases in the frequency and diversity of short bursts of spontaneous activity. Developmental desynchronisation of spontaneous network activity has been demonstrated using Ca\(^{2+}\) imaging (i.e. recorded with slower timescale but greater single-cell resolution in visual (Rochefort et al., 2009) and somatosensory (Golshani et al., 2009) cortices at eye opening and the onset of whisking, respectively.

1.6.6.8. GABAergic maturation and Layer 4 cortical circuit function

Minlebaev et al. (2011) reported a developmental shortening in the relative lag between the onset of network-sustained EPSCs and IPSCs in Layer 4 from \textit{in vivo} recordings – presumably mediated by the facilitated recruitment of local interneurons due to increased Ex-In neuron connectivity. The authors did not specifically address the relative amplitudes or short-latency timing of these current components in Layer 4. However fast and proportionate feedback inhibition has been shown previously to be an emergent property of mature cortical and hippocampal networks (Oren et al., 2006; Okun and Lampl, 2008; Atallah and Scanziani, 2009; Haider and McCormick, 2009; Akam et al., 2012; Haider et al., 2012) that critically supports diverse, putatively scale-free propagation of network firing patterns (Beggs and Plenz, 2003; Vogels et al., 2005; Shew et al., 2011). Gireesh and Plenz (2008) reported that critical (NMDA and GABA- dependent) avalanche activity in Layer 2/3 of barrel cortex emerges developmentally as “nested theta and beta/gamma oscillations”.

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Interestingly, these effects were dependent on (D1) dopaminergic transmission, but not gap junctions (important in the synchronization of electrically coupled cortical GABAergic interneurons; Parker et al. 2009) or cholinergic modulation (involved in cortical arousal and modifying sensory responses; Hsieh et al. 2000; Constantinople and Bruno 2011). Moreover Colonnese et al. (2010) reported that severing noradrenergic inputs from the brainstem could rescind the developmental changes in visual evoked responses discussed above. Overall, Gireesh and Plenz (2008) hypothesize that the precise orchestration of gamma oscillatory activity during development facilitates the organization of spatiotemporally diverse synchronized network patterns. It should be noted that the temporal resolution of multi-unit correlations reported in Gireesh and Plenz (2008) is higher than that at which a developmental desynchronization of spontaneous network activity was demonstrated in visual (Rochefort et al., 2009) and somatosensory (Golshani et al., 2009) cortices at eye opening and the onset of vision, respectively. These latter results are also more representative of circuit activity in Layer 2/3, due to imaging constraints. A more recent study on the developmental profile of whisker-evoked pairwise correlations in sensory evoked Layer 4 firing responses was performed by Ikezoe et al. (2012). The authors reported little change to the response variability of single neuron firing patterns between P5~30, but a gradual reduction in the co-variability between pairs of neurons, suggesting an overall increase in the barrel-wide precision of sensory responses takes place over a protracted developmental period.
1.6.6.9. Physiological functions of the L4 circuit

Finally, what physiological computations does Layer 4 perform? This remains unclear, however leading theoretical predictions are summarised in Table 1-1:

<table>
<thead>
<tr>
<th>Proposed function</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing redundancy in inputs</td>
<td>(Barlow, 1989)</td>
</tr>
<tr>
<td>Extracting low-order directional derivatives of inputs</td>
<td>(Adelson and Bergen, 1991)</td>
</tr>
<tr>
<td>Input-output information maximization</td>
<td>(Linsker, 1993; Okajima, 2001)</td>
</tr>
<tr>
<td>Preservation of spatial relationships in the input</td>
<td>(Li and Atick, 1994)</td>
</tr>
<tr>
<td>Efficient sparse coding</td>
<td>(Bell and Sejnowski, 1997; Olshausen and Field, 1997, 2004; Rehn and Sommer, 2007; Evans and Prescott, 2013)</td>
</tr>
<tr>
<td>Temporal coherence maximization</td>
<td>(Hurri and Hyvärinen, 2003)</td>
</tr>
<tr>
<td>Tuning to particular input patterns as a step to generalization</td>
<td>(Poggio and Bizzi, 2004)</td>
</tr>
</tbody>
</table>

**Table 1-1** Computations proposed to be performed by Layer 4 of primary visual and somatosensory cortex. Also see Miller et al., 2001; Favorov and Kursun, 2011 for discussion).

Recent physiological evidence also suggests that in primary sensory cortex Layer 4 acts to amplify and prolong the response duration of successfully filtered thalamic inputs (Li et al., 2013a, 2013b; Lien and Scanziani, 2013), and that faithful representation of their statistics by the recurrent circuit
response is crucial for maintaining this internal representation of sensory context for downstream cortical processing. However, trial-to-trial variability is observed in the pairwise synchrony and patterns of sensory-evoked firing in Layer 4 neurons (Khatri et al., 2009). A substantial fraction of this is independent of input statistics, and emerges in the cortex suggesting that the correct balance between correlated and sparse firing is an important parameter for sensory representation in cortical circuits. In the sparse coding framework proposed by Barlow (Barlow 1972, discussed more recently by Olshausen and Field 2004; Wolfe et al. 2010), highly tuned stimulus responsivity in cortical microcircuits builds hierarchically, such that sequentially more complex responses are projected between successive levels of processing. In particular, Olshausen and Field suggest that in primary sensory cortex, an overcomplete “dictionary” of neurons providing filter functions tuned to respond to specific patterns of synaptic activity is beneficial for reducing dimensionality of natural input scenes and efficiently maximizing information transmission (Figure 1-4). This has recently found support in experimental and theoretical work (Jadhav et al., 2009; Wolfe et al., 2010; Favorov and Kursun, 2011; Evans and Prescott, 2013).

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12 Referenced elsewhere in the text, but summarized in (Feldmeyer et al., 2012)
**Figure 1-4** Schematic of how dimension reduction is thought to be performed in Layer 4 processing by an overcomplete sensory feature representation set.

Black lines are the preferred sensory features of each neuron receiving thalamic input. Blue ellipses are tiled feature-responsive fields of each neuron (i.e. it will fire in this zone). The red curve is the hypothetical trajectory of a sensory scene (e.g. principle whisker moving across an edge) as it would appear translating across the input array. A sufficiently dense (i.e. overcomplete: more possible pattern vectors than input dimensions) tiling of responsive fields adequately describes a highly curved trajectory (that is, a complex scene) by ensemble firing patterns, here seen flattened as would be observed at higher stages of analysis. Adapted for the whisker system from (Olshausen and Field, 2004).
1.6.7. Fragile X Syndrome

1.6.7.1. Background and Significance

Affecting one in 3000 male and one in approximately 6000 female children, FXS is the most common form of hereditary Intellectual disability and a leading genetic model of Autism. Up to a third of FXS patients are diagnosed as autistic, while <2% of autistic spectrum diagnosed children are comorbid for FXS and 97% of patients are comorbid with intellectual disability (O'Donnell and Warren, 2002). Unlike many forms of idiopathic Autism, The syndrome is heritable and monogenic in origin: a trinucleotide expansion in the \textit{Fmr1} gene leads to hypermethylation and subsequent transcriptional silencing, with the loss of the protein product, FMRP (Grossman et al., 2006). The genomic distortion is visible in the karyotype of FXS patients as a nick in the long arm of the X chromosome (below, hence the “Fragile” moniker).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures/Figure1-5.png}
\caption{Biological features of FXS}
\end{figure}

\textbf{a).} The “Fragile” X chromosome. Electron Micrograph redrawn from (Harrison et al., 1983). \textbf{b).} Sections of Golgi-impregnated dendrite from FXS (left) and normal (right) human cortical neurons. Redrawn from (Irwin et al., 2000).
FMRP is a RNA binding protein implicated in regulation of activity-dependent protein synthesis and mRNA trafficking, (below, for review see (Hagerman and Hagerman, 2002) as well as regulating mRNA stability (Zalfa et al., 2007).

![Figure 1-6 Trafficking of mRNA by FMRP. Redrawn from (Bagni and Greenough, 2005).]

In addition to specific peripheral abnormalities, FXS patients present with a battery of neuropsychiatric symptoms, including severely impaired delayed cognitive development, impaired circadian rhythm and sleep deprivation, epilepsy, altered EEG recordings, sensory hypersensitivity and autistic characteristics (reviewed by Belmonte and Bourgeron, 2006). Notably for this study, FXS patients demonstrate impaired sensorimotor gating (Frankland et al., 2004). Ostensibly, such a spectrum of cognitive and developmental defects is hard to reconcile with a single genetic abnormality, however many can be linked to improper development and refinement of neocortical circuitry. Additionally, it is plausible that the
diversity of syndromic phenotypes in FXS is related in turn to the functionally diverse roles that FMRP plays in regulating cortical development.

The brains of FXS patients are grossly normal, but despite a shortage of post-mortem tissue, analyses across several cortical areas demonstrate a hallmark preponderance of thin, tortuous dendritic spines (Figure 1-5b) - the functional postsynaptic units of excitatory synaptic transmission - at early postnatal and adolescent ages (Hinton et al., 1991).

1.6.7.2. The Fmr1-KO mouse

A mammalian model of FXS is provided by the Fmr1-KO mouse, in which Fmr1 is genetically deleted (Consortium, 1994). The monogenic nature of this model and reproducibility of human disease phenotypes (Churchill et al., 2002), combined with the strong link between FXS and Autism, have led to the Fmr1-KO mouse’s current status as a leading animal model for Autism (Bernardet and Crusio, 2006).

Using this mouse model, numerous approaches have been employed to study the origins of molecular, anatomical and physiological defects in FXS. The abnormal dendritic spine phenotype observed in FXS patients is replicated in Fmr1-KO mice, agreeing with the spine morphology observed in neurons of immature brains (Dunaevsky et al., 1999) and those subjected to sensory deprivation (Lendvai et al., 2000). An additional role for FMRP in the timely stabilization of dendritic spines has also been recently proposed (Cruz-Martin et al., 2010). Crucially in Fmr1-KO mice, aspects of this phenotype decline with age, such that the morphology and density of
dendritic spines in some neurons of adult brains are indistinguishable from those of normal (littermate) controls (Nimchinsky et al., 2001; Galvez and Greenough, 2005). This is of particular significance to the developmental delay in FXS patients.

Additional anatomical defects have been reported for experience-dependent specific dendritic arborisation (Galvez et al., 2003; Till et al., 2012), suggesting a role for FMRP in selective developmental pruning of dendrites. These results coupled with the observation that FMRP protein levels are upregulated in response to somatosensory (Todd et al., 2003) and visual (Gabel et al., 2004) stimuli strongly implicate FMRP in activity-dependent synaptic maturation. Specifically, a requirement for sensory experience in normal spinogenesis (Lendvai et al., 2000) and orientation-selective dendritic arborisation (Verley and Axelrad, 1977) of sensory cortical neurons suggests deficient production and/or maintenance of appropriate synaptic connections in the FXS cortex. To understand whether these changes in activity-dependent synaptic plasticity within the context of anatomical defects in Fmr1-KO mice, a priority will be to perform correlated structural and functional investigations.

In Fmr1-KO mice, defects in dendritic and spine anatomy and altered synaptic plasticity are functionally linked by their converging altered regulation at the postsynaptic density (PSD), the functional postsynaptic protein complex regulating dendritic spine morphology and synaptic function in excitatory neurons. Work in identifying the mRNA targets of FRMP has elucidated the wide range of genes whose translational control is impaired in its absence (Darnell et al., 2001; Jensen and Darnell, 2008).
Electrophysiological investigations in Fmr1-KO mice have demonstrated abnormal synaptic plasticity, particularly forms of long-term potentiation and depression (LTP and LTD, respectively). At hippocampal synapses in the normal brain, stimulation of postsynaptic group 1 metabotropic glutamate receptors (Gp1 mGluRs) leads to a transient depression of synaptic transmission, via internalization of AMPA and NMDA glutamate receptors (Oliet et al., 1997), which requires novel protein synthesis at subsynaptic loci to achieve permanence (Bender et al., 2006). This “mGluR-LTD” can also cause spine shrinkage and elongation (Laumonnier et al., 2007). FMRP binds a subset of dendritic mRNAs and functions through feedback inhibition to repress their translation (Fox, 2002).

Given that Fmr1 is included in the subset of mRNAs whose translation are stimulated by mGluR activation (Weiler et al., 1997), it has been proposed that loss of FMRP leads to exaggerated mGluR-LTD in the absence of this translational inhibition. This is supported by excessive mGluR-dependent AMPA receptor internalization in the absence of FMRP (Nakamoto et al., 2007). Early postnatal Fmr1-KO brains show robust (somatosensory: Desai et al., 2006) or enhanced (hippocampal: Huber et al., 2002) mGluR-LTD but altered NMDA-dependent LTP (Meredith et al., 2007a). As mGluR-LTD is capable of irreversibly de-potentiating homosynaptic NMDA receptor-dependant LTP (Oliet et al., 1997), it is plausible that excessive mGluR-LTD prevents maturation of spine morphology by preventing NMDA receptor-dependent potentiation of synapses.

These findings were formally synthesised into the “mGluR theory of Fragile X” (Bear et al., 2004), shown in Figure 1-7. The Bear lab explicitly
tested this theory by showing rescue of many FXS-related *Fmr1-KO* phenotypes by genetically reducing activity of mGluR5 (Dölen et al., 2007). Subsequently, it was shown that pharmacological reduction of mGluR5 activity in adult mice could also produce good cognitive improvement (Michalon et al., 2012). Translating to the clinic, current leads include Mavoglurant (AFQ-056), an mGluR5 antagonist and Fenobam, a negative allosteric modulator of mGluR5 (Berry-Kravis et al., 2009; Levenga et al., 2010, 2011; Hagerman et al., 2012). Further examples of currently proposed targeted treatment strategies include modulating GABA<sub>b</sub> receptors (Henderson et al., 2012), and promoting maturation of dendritic spines using minocycline (Bilousova et al., 2009; Schneider et al., 2013).

Figure 1-7 Theories of FMRP regulation of synaptic function.

**a).** The original “mGluR” theory (Bear et al., 2004). **b).** Updated to include additional regulation by Tsc1/2 (Auerbach et al., 2011)

These compounds represent targeted treatments of the underlying disorder, rather than associated symptoms of FXS, which are currently managed with a combination of antidepressants (SSRIs), atypical antipsychotics and stimulants (reviewed in Hagerman et al., 2009), and
behavioural therapy (Cascio, 2010). It is clear however, that our current understanding of circuit development and maintenance in the FMRP-null brain is far from complete. Recent research has widened to appreciate the interplay between neuronal anatomy and intrinsic physiology, circuit function and synaptic plasticity (notably reviewed in Lee and Jan, 2012; Portera-Cailliau, 2012, and addressed in more detail below).

For these reasons, it is essential to study early critical period defects: understanding dynamic circuit maturation could help explain many subsequent anomalies in sensory processing observed in older Fmr1-KO mice and FXS patients (Laumonnier et al., 2007). In order to examine the role of FMRP in early sensory experience-dependent plasticity and anatomical development, it is necessary to systematically examine the well-characterized developmental processes that take place during these early postnatal periods. As circuit formation in Layer 4 precedes development of more superficial cortical layers and is heavily influenced by sensory activity, it represents a logical target for investigation upon which to build an understanding of subsequent ‘downstream’ developmental steps in sensory cortical dysfunction.

1.6.7.3. Pathophysiological changes in intrinsic neuronal excitability in FXS

The homeostasis of neuronal input-output transformations within a useful working range is a task attributed to plasticity of voltage-gated ion channels (Nelson and Turrigiano, 2008)
Potentially far-reaching single-cell and network-wide physiological disruptions of neuronal input-output transformations can result from ostensibly small changes in the intrinsic properties of neuronal membrane excitability (best described for epilepsy, e.g. Poolos and Johnston 2012; Benarroch 2013). It is particularly important to consider how such changes affect responsivity to network inputs during sensitive developmental periods for circuit plasticity. A case in point: in the Fmr1-KO mouse, Strumbos and colleagues demonstrated a flattened tonotopic gradient and absence of experience-dependent plasticity in the auditory brainstem (MNTB) that involved defects in the control of Kv3.1 mRNA expression (Strumbos et al., 2010). Such a defect is likely to hamper the ability of fast-spiking MNTB neurons to faithfully report stimuli across the full auditory range, and could contribute to auditory hypersensitivity and speech problems in FXS (Abbeduto and Hagerman, 1997; Miller et al., 1999), and by extension interfere with both language acquisition and social interaction, as well as learning and attentional control.

A rank of the top 80 strongest FMRP-associating mRNAs is included in Appendix 1 (Brown et al., 2001). Ion channels (e.g. HCN2, rank=10, L-type Ca\(^{2+}\) channel subunits, rank=31) and accessory subunits (e.g. Na\(^{+}\) channel-related protein, rank=46) feature heavily in the list of gene products whose expression are misregulated at the mRNA level in the absence of FMRP. More recently, two intriguing reports have demonstrated novel direct protein-protein interactions between FMRP and surface-expressed ion channels (Brown et al., 2010; Deng et al., 2013) in what could be a diverse
and powerful regulatory role for FMRP in the normal brain, but a further confound to understanding the pathobiology of FXS.

1.6.7.4. Interactions between altered intrinsic excitability and impaired plasticity mechanisms in FXS

A number of studies in knockout animals have explored whether loss of FMRP affects intrinsic neuronal membrane properties. Table 1-2 summarises the main findings of these papers. Several reports that demonstrate altered expression of voltage-gated ion channels rate also show abnormal plasticity mechanisms such as LTP, STDP or homeostatic mechanisms. These have been interpreted as at least partly affected as a consequence of altered cell-intrinsic properties (Lee et al., 2011b; Lee and Jan, 2012) - the converse is not always the case (e.g. Desai et al. 2006; Meredith et al. 2007)). Meredith and Mansvelder (2010) note that while changes to functional neuronal anatomy reported in the literature are small, (typically 10-20% changes in baseline synaptic transmission and dendritic spine density, (e.g. Desai et al. 2006; Pfeiffer and Huber 2007)), large changes to synaptic plasticity mechanisms are characteristic, both in FXS and other forms of ID. The authors discuss potential interactions among anatomical and plasticity phenotypes of FXS and consider a compounding role of altered dendritic excitability, focusing discussion on changes to Spike-Timing-Dependent Plasticity (STDP). This group have previously shown that STDP was affected in the Fmr1-KO prefrontal cortex such that the postsynaptic activity threshold for induction of timing-dependent potentiation was raised, a factor attributed to failed back-propagating action potentials (bAPs) and unreliable calcium signalling.
Such a finding suggests that altered dendritic excitability may contribute to bAP failures.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Brain area (Age, cell)</th>
<th>Effect on physiology</th>
<th>Mechanism implicated</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Fmr1-KO mouse | Hipp. CA1 (Adult Excitatory) | • Lower dendritic $R_{input}$  
• Higher resonant frequency  
• Weaker temp summation  
• Impaired homeostatic plasticity | Dendritic $I_h$ (HCN1) | Brager et al. 2012 |
| Fmr1-KO mouse | Olf. bulb, brainstem (Adult Excitatory) | • Reduced SLACK density  
• Altered SLACK activity | $K_{Na}$ (SLACK) | Brown et al. 2010 |
| Fmr1-KO mouse | Hipp. CA3-CA1, L5 cortical (Adult Excitatory) | • AP broadening  
• Elevated Ca$^{2+}$ entry  
• Altered STP & information transmission | High-threshold Ca$^{2+}$ channels (BK) | Deng et al. 2013 |
| Fmr1-KO mouse | L5 cortical (P10-18 Excitatory) | • Unaltered intrinsic excitability  
• Altered STDP | ? | Desai et al. 2006 |
| Fmr1-KO mouse | L4 cortical (P14-P28 Excitatory, Inhibitory) | • Elevated excitability (EX neurons)  
• Lower RMP (FS interneurons)  
• Altered connectivity | ? | Gibson et al. 2008 |
| Fmr1-KO mouse | Hipp. CA1 (Adult Excitatory) | • Elevated $K_{4.2}$ expression  
• Loss of LTP | $K_{4.2}$ | Lee et al. 2011 |
| Fmr1-KO mouse | mPFC (Adult Excitatory) | • Unaltered intrinsic excitability  
• Altered STDP  
• Reduced Ca$^{2+}$ entry | L-type Ca$^{2+}$ channels | Meredith et al. 2007 |
| Fmr1-KO mouse | Basolateral Amygdala (Adult Excitatory) | • Elevated intrinsic excitability  
• Reduced tonic inhibition | Tonic inhibition | Olmos-Serrano et al. 2010 |
| Fmr1-KO2 mouse | Hipp. CA1 (P14, adult) | • Unaltered intrinsic excitability  
• Elevated NMDA/AMPA ratio | Glutamatergic ionotropic transmission | Pilpel and Kolleker 2009 |
| Fmr1-KO mouse | Brainstem (Adult Excitatory) | • Flat tonotopic gradient in MNTB  
• Reduced tone-induced plasticity | $K_{3.1}$ | Strumbos et al. 2010 |
| dfmr1 fly | Mushroom Body, Excitatory | • Exaggerated [Ca$^{2+}$] elevation upon depolarization | Calmodulin Calbindin | Tessie and Broadie 2010 |
| Aplysia w/ FMRP inactivation | Bag Cell | • Elevated resting potential  
• Reduced input resistance | $K_{Na}$ (SLACK) | Zhang et al. 2012 |

**Table 1-2** Literature summary highlighting phenotypes in animal models of FXS associated with altered intrinsic neuronal excitability.
Dendritic arborisation and spine abnormalities (Kaufmann and Moser, 2000; Bagni and Greenough, 2005), altered voltage-gated calcium channel conductances (Chen and Toth, 2001; Meredith et al., 2007b; Tessier and Broadie, 2011; Deng et al., 2013), altered potassium channel conductances (Brown et al., 2010; Strumbos et al., 2010; Brager et al., 2012; Zhang et al., 2012) and changes in GABAergic tone (D’Hulst et al., 2006, 2009; Gibson et al., 2008; Olmos-Serrano et al., 2010; Vislay et al., 2013) have all been demonstrated in Fmr1 null circuits and could contribute to altered STDP through altering the timing and magnitude of calcium rise upon dendritic bAP invasion.

Lee and Jan (2012) further develop this argument by noting that the LTP deficits characteristic of Fmr1-KO neurons (e.g. Lauterborn et al. 2007; Shang et al. 2009; Chen et al. 2010) cannot be rescued solely by reducing mGluR5 activity (e.g. with MPEP: Wilson and Cox 2007; Suvrathan et al. 2010, for review see Hagerman et al. 2012) and suggest that modulation of K\textsuperscript{+} channel activity may be necessary or beneficial as an adjunct therapy.

Recent work now directly links altered dendritic voltage-gated K\textsuperscript{+} conductances to defective LTP in FXS. In an elegant study, Lee and colleagues (Lee et al., 2011b) demonstrated that dendritic targeting and translational repression of K\textsubscript{v}4.2 mRNA by FMRP is responsible for the dendritic expression of K\textsubscript{v}4.2 in hippocamal pyramidal dendrites in the normal brain. This spatial gradient both dendritically compartmentalises voltage summation (Losonczty et al., 2008) and limits the retrograde dendritic and spine invasion by bAPs (first demonstrated by Hoffman et al. 1997). Either raising (Jung et al., 2008) or ablating (Chen et al., 2006) K\textsubscript{v}4.2
abolishes or increases hippocampal NMDAR-dependent postsynaptic LTP respectively.

Lee and colleagues (2011a) show excessive Kv4.2 expression in Fmr1-KO hippocampal pyramidal cell dendrites and find that blocking Kv4.2 with heteropodatoxin restores normal levels of LTP induction in mutant tissue. This study also provides interesting insight into homeostatic regulation of dendritic excitability and bidirectional interaction between NMDAR-LTP and Kv4.2 expression (Jung et al., 2008). NMDAR-dependent LTP in CA1 leads to internalisation and degradation of Kv4.2 channels (e.g. Kim et al. 2007). What was previously unclear was how re-expression of Kv4.2 could bounce back rapidly after NMDAR-mediated degradation. Lee and colleagues suggest that transient de-repression of low baseline (FMRP-repressed) Kv4.2 mRNA expression results from NMDAR-triggered FMRP de-phosphorylation (likely by protein phosphatase 1). Such an antagonistic increase would counteract simultaneous NMDAR-dependent Kv4.2 degradation and predicts only a transient down-regulation of Kv4.2, in turn maintaining intrinsic excitability and maintaining a dynamic range for subsequent plastic changes.

Most recently, a direct link between altered dendritic HCN channel expression, and intrinsic plasticity has been demonstrated in Fmr1-KO hippocampal CA1 neurons (Brager et al., 2012). HCN channels provide the hyperpolarisation-activated current Ih, active near resting potential and a crucial determinant of input resistance and neuronal voltage behaviour near resting membrane potential (Magee, 1998). The authors find a significantly enhanced dendritic Ih current density in Fmr1-KO neurons,
and a corresponding drop in dendritic input resistance and frequency-shifted impedance profile. The effect on temporal integration was as predicted, with weaker temporal summation of EPSPs observed at plausible frequencies of synaptic input. More striking however, whilst mGluR5-dependent persistent depressive reductions in $I_h$ were intact in mutant neurons, persistent potentiating increases in $I_h$ density could not be achieved after theta-burst induction. This finding further links defects in baseline neuronal function brought about by altered intrinsic properties to a use-dependent plasticity defect that by affecting synaptic integration could alter network performance.

1.6.7.5. Manifestation of intrinsic neuronal properties in disrupted network activity in FXS

At the circuit level, a large body of work now implicates an altered balance of Inhibition/Excitation synaptic transmission in FXS pathophysiology (discussed in detail in chapters 4 and 5, reviewed in D’Hulst and Kooy 2007; Brooks-Kayal 2010; Paluszkiewicz et al. 2011; Coghlan et al. 2012). The extent to which this input imbalance influences neuronal firing rates and timing of spikes will be influenced by cells’ intrinsic excitability. This link has been under-explored in the literature and should be considered important to therapeutic strategies, as will be expanded upon in this and subsequent chapters. For example, in the barrel cortex at P14 and P28, Gibson and colleagues found reduced In/Ex ratios in circuit-evoked input to excitatory neurons, as well as strong reduction in connectivity between excitatory and inhibitory neurons (Gibson et al., 2008). The elevated input resistance additionally reported specifically in excitatory but not inhibitory
neurons in this study could contribute to further tipping the balance of circuit activity towards runaway excitation. Longer cortical UP states reported in this and a subsequent study from the same group (Hays et al., 2011) and also the present work (see chapter 5) likely reflect this. Further support for distortion of circuit activity by altered intrinsic excitability comes from recent finding from the Portera-Cailliau laboratory, in which young *Fm1-KO* barrel cortex neurons showed greater participation in, and fired more APs during spontaneously arising UP states recorded whilst resting or sleeping (Gonçalves et al., 2013). Conversely, little difference was observed during brain states associated with arousal and wakefulness (“Fast Oscillatory Activity”, FAO). The authors present data suggesting that correlated firing activity is increased during UP states, however it is known that correlations between spike trains increase with the neurons’ mean firing rate (de la Rocha et al., 2007), potentially confounding this interpretation. By this logic, the reported increased intra-network correlation could be a secondary effect of elevated firing rates brought about by neuronal gain increases due to elevated neuronal input resistance. If so, altered neuronal impedance spectra (i.e. more aggressive high-cut filtering) would be expected, favouring slow oscillatory activity over fast, gamma-band activity. This interpretation is consistent with the lack of changes to spiking activity recorded during FAO in Gonçalves et al. 2013, however the authors show no change in relative spectral power densities of ‘high’ vs. ‘low’ power ratios from EEG. Nonetheless, FXS patients show markedly disrupted EEG, correlating with impaired sleep cycles (Miano et al., 2008). Furthermore, theta power (4-8Hz) is increased relative to high-
alpha (10-12Hz) power in FXS patients during resting-state EEG recordings (Van der Molen and Van der Molen, 2013). Changes to the relative spectral power in higher mu (associated with locomotor planning, execution and imitation, e.g. Bernier et al. 2007) and gamma (associated with attention and sensory processing, e.g. Brown et al. 2005; Orekhova et al. 2007; Sun et al. 2012) bands have also been reported, as well as reduced functional connectivity (Rippon et al., 2007). Precise oscillatory timing between populations of neurons in one brain area when projecting coherent input to a second, target area is essential to prevent signal distortion (Akam and Kullmann, 2010, 2012). It is a clear prediction therefore that frequency responsiveness distorted by altered intrinsic impedance can be expected to rapidly degrade such ‘communication through coherence’ channels by destroying coherence (for stimulating discussions of the CTC hypothesis, see: Varela et al. 2001; Womelsdorf et al. 2006; Fries 2009; Roberts et al. 2013).

1.6.7.6. Critical period disruption in the Fmr1-KO mouse

Factors contributing to the duration and closure of critical periods are diverse and likely involve interaction of numerous genetic and experience-dependent factors. A key study in Fmr1-KO mice from the Contractor and Kind laboratories (Harlow et al., 2010) demonstrated that in wild-type mice, expression of FMRP closely follows the critical period for induction of synaptic plasticity at thalamocortical (TC) synapses (between Ventrobasal thalamic (VPM or VB) neurons and cortical Layer 4 spiny stellate neurons). Thus FMRP expression increases during the first
postnatal week, peaking at the close of the critical period (P7) before declining to baseline levels by P14. This was further paralleled by the strength of the NMDA-mediated contribution to the TCA-induced EPSC. The authors found an unusually small NMDA-receptor component at the start of what is normally the critical period (P4), which then increased allowing robust plasticity past P7, before decreasing to normal levels by P9. Correspondingly, the critical period for LTP induction at thalamocortical synapses was similarly delayed. This is further supportive of previous findings in which the strengthening of ascending connections from Layer IV to II/III was shown to be delayed in the Fmr1-KO mouse until P21 (Bureau et al., 2008); a finding due in part to spatially diffuse axon termini projecting into Layer II/III, a reduced phenotype by 3 weeks. More recently, a study reported altered critical period maturation of tonotopic maps in auditory cortex (Kim et al., 2013). Interestingly, this phenotype was rescued by daily injection of the mGluR5 antagonist MPEP, suggesting that this pharmacological strategy can be effective at improving abnormal critical period developmental plasticity.

1.6.7.7. GABAergic dysfunction in Fmr1-KOs

In addition to abnormal glutamatergic signalling in Fmr1-KO mice, widespread defects in GABAergic transmission are also observed. Neocortical inhibitory circuits are essential for faithful and balanced sensory processing (Uhlhaas and Singer, 2010) and play crucial roles in developmental plasticity (Hensch, 2005). A reduction in free GABA, likely caused by a substantial reduction in GAD67 expression (D’Hulst et al.,
2006) has been linked to impaired GABAergic transmission in the Fmr1-KO hippocampus (D'Hulst and Kooy, 2007), while reduced expression of multiple GABA$_{	ext{A}}$ subunits have been reported at mRNA level and in protein extracts (GABA$_{	ext{A}}$ β-subunit). An altered laminar distribution of Parvalbumin-positive interneurons; accounting for the majority of fast-spiking interneurons, was also reported in Fmr1-KO mouse cortex (Selby et al., 2007). These findings provide a tenable link to the epileptogenic phenotype of FXS and, given the proposed role of Layer 4 inhibitory circuitry in gating translaminar propagation of afferent sensory information for subsequent processing (Rozas et al., 2001), suggest an additional mechanism, potentially explaining symptoms of sensory hyperacuity.

Additionally, like those surrounding excessive mGluR activation, these findings prompt insight towards a potential pharmacological intervention. However, pathophysiological GABAergic circuitry has yet to be fully characterized, and their role in aberrant cortical development is as of yet unclear. Therefore a full description of these developmental defects in the inhibitory system with regards to perturbed activity-dependent processes in FXS represents a pressing requirement.

Studying GABAergic defects at the circuit level in the Fmr1-KO mouse, Gibson and co-workers (Gibson et al., 2008) found a marked reduction of local excitatory drive onto fast-spiking inhibitory interneurons (FS neurons) in barrel cortex Layer 4, while normal monosynaptic GABAergic transmission onto excitatory stellate cells was observed. This is significant:

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13 Although the Kind laboratory have not been able to repeat these findings
upon excitation by thalamic sensory input, stellate neurons excite local FS cells. FS cells in turn project recurrent connections back to the stellate cells. Together, this tri-synaptic arc mediates local feedback inhibition, crucial to constraining levels of recurrent excitation in local circuits to within acceptable levels. Thus, in FraX mice the decrease in stellate to FS cell drive contributed to a pronounced deficit in feedback inhibition in Layer 4, and a decrease in circuit inhibition that led to longer “UP” states; representative of network hyperexcitability. The Gibson lab subsequently showed that the abnormal UP states in Fmr1-KO slices emerged from defective excitatory circuitry (Hays et al., 2011). Here, genetically deleting FMRP in interneurons had little effect on UP state duration, whilst targeted deletion in excitatory cortical neurons produced an effect similar to the constitutive Fmr1-KO knock-out. Furthermore, the authors reported rescue of UP state duration in Fmr1-KOs with pharmacological blockade, or genetic reduction of mGluR5 activity. An elegant follow-up study used genetically-targeted paired recordings between Layer 4 neurons in a “mosaic” Fmr1-KO mouse (Patel et al., 2013). The deficit in Ex-FS connectivity was specific to pairs in which the presynaptic Ex cell lacked FMRP, and mediated by a reduction in glutamate release probability. No such effect was observed in Ex-Ex pairs. Interestingly, these findings were also repeated using paired recordings in other cortical layers, suggesting that the deficit in GABAergic connectivity was not specific to Layer 4.
2. Materials and Methods

2.1. Animals and tissue

2.3.1. Strains and breeding of mice

*Fmr1*-KO mice were obtained from the Contractor lab (Northwestern University, Chicago IL) and maintained (*Fmr1*+/− females crossed with wild-type males) on a congenic C57Bl6/J background. Animals had access to food and drinking water *ad libitam*. All procedures were carried out in accordance with NIH and IUPAC (USA) and UK Home Office guidelines.

2.3.2. Genotyping

For colony maintenance, mice were ear-punched at P14, for post-mortem genotyping, a section of tail tissue was taken. Standard genotyping procedures were followed. Primers used are shown below.

**Wild-type reaction**, amplifies a ~500bp product from the wild-type allele:

- 2009: 5'- GTG GTT AGC TAA AGT GAG GAT GAT -3'
- 2010: 5'- CAG GTT TGT TGG GAT TAA CAG ATC -3'

**Knockout reaction**, amplifies a 197bp fragment of the Neomycin cassette:

- 162: 5'- CCG GTT CTT TTT GTC AAG ACC G -3'
- 163: 5'- CGG CAG GAG CAA GGT GAG AT -3'

2.3.3. Preparation of acute brain slices

Thalamocortical brain slices were prepared following (Agmon and Connors, 1991), with modifications reported in (Daw et al., 2007a; Chittajallu and Isaac, 2010). Briefly, animals were decapitated without
anaesthesia (at ages younger than P14, or eyelid separation, if earlier). Brains were rapidly removed and placed in carbogenated (i.e. bubble to saturation with 95% O₂/5% CO₂) ice-cold cutting solution. A cut was made parallel to the thalamocortical tract (approximately from juncture between cortex and cerebellum) at 40° from the midline (ages <P10). Due to age-dependent anatomical changes, the cut angle was increased to ~45° from P11 onwards, with the scalpel blade additionally inclined ~10° away from the vertical. Excess posterior tissue was trimmed from the hemisected brain, and the brain section was flipped onto the edge of the first cut, and attached to the slicing platform with cyanoacrylate glue. Brains were sliced in carbogenated ice-cold cutting solution using a Leica VTS-1000 slicer. 400μm sections were prepared. Sections were examined under brightfield/transmitted visible light microscopy. Thalamocortical fibres are easier to distinguish in older tissue, due to the onset of myelination, whereas barrel boundaries become harder to determine due to a reduction in visual contrast from ongoing cortical development. Sections containing VB thalamus and barrel cortex, as well as thalamocortical fibres were retained and transferred to carbogenated holding chamber containing artificial cerebrospinal fluid (aCSF) at room temperature. Slices were allowed to recover for 1 hour post slicing, after which they were typically usable for 5-7 hours, although the anatomical quality was observed to degrade over time.
2.3.4. Electrophysiological solutions

2.3.4.1. aCSF

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>119</td>
</tr>
<tr>
<td>KCl</td>
<td>2.5</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>1</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>26.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>11</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>9/1.3*</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>5/2.5*</td>
</tr>
</tbody>
</table>

*Slicing/recording concentrations. Osmolality adjusted to 305mOsm, saturated with 95% O$_2$/5% CO$_2$, pH7.4 at 31-32°C.

2.3.4.2. Whole-cell internal solutions

The majority of experiments, unless otherwise specified, were performed with the following K-based pipette internal solution:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMeSO$_4$</td>
<td>130</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.5</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.5</td>
</tr>
<tr>
<td>Mg-ATP</td>
<td>4</td>
</tr>
<tr>
<td>Na-GTP</td>
<td>0.3</td>
</tr>
</tbody>
</table>

For voltage-clamp data reported in 5.3.3, the following Cs-based solution was used to improve space clamp during network activity:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsMeSO$_4$</td>
<td>130</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.5</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.5</td>
</tr>
<tr>
<td>Mg-ATP</td>
<td>4</td>
</tr>
<tr>
<td>Na-GTP</td>
<td>0.3</td>
</tr>
<tr>
<td>QX-314</td>
<td>5</td>
</tr>
</tbody>
</table>
Osmolality adjusted to 285mOsm, saturated with 95% O₂/5% CO₂. pH7.4 at 31-32°C. Junction potential =9.1mV (K⁺) / 8.4mV (Cs⁺). E_{rev} Cl⁻=-71mV @31°C. Biocytin (if included) was added to final concentration of 3mg/ml. Reported membrane potentials are uncorrected for junction potential.

All chemicals and drugs were obtained from Sigma and Tocris, respectively.

2.3.5. Fixation and biocytin immunohistochemistry

For post-hoc visualisation of neuronal anatomy, slices were drop fixed in 4% paraformaldehyde in pH6.8 phosphate buffer (PB) overnight at 4°C, washed twice with PB and transferred to pH 7.4 phosphate buffered saline (PBS) for (short-term) storage.

To reveal anatomy of previously patched neurons, slices were incubated overnight (room temperature, shaking) in a reaction buffer containing 1:250 Alexa-568 conjugated Streptavidin (Invitrogen), 50μM NH₄Cl, 0.2% Triton X-100, in PBS. Slices were washed 3x with PBS and stored in PBS/0.001% Sodium Azide, protected from light at 4°C.

2.3.6. Confocal imaging and anatomical reconstruction

Slices were de-salted in double-distilled H₂O for 24h prior to transferring to mounting medium. 12-24h prior to imaging, slices were transferred to soft-set Vectashield (Vector labs).

Slices were wet mounted in Vectashield by sandwiching between a cover glass and the bottom (glass) of a “POC” chamber designed for live-cell
imaging. Imaging was performed using a Zeiss LSM 510 inverted confocal microscope, using a 543nm He:Ne laser line and a 20x/0.8NA Olympus objective.

Image stacks were stitched in FIJI/ImageJ and imported into NeuronStudio (Rodriguez et al., 2008) for semi-automated arbour tracing. For modelling performed in section 3.4.8, Neuronconstruct (Gleeson et al., 2007) was used to add passive electrophysiological parameters (membrane leak conductance and capacitance), as well as model synapses, before exporting to the Neuron environment for simulation.

2.4. Patch-clamp recordings from cortical neurons

For patch-clamp recordings, slices were transferred to the recording chamber of an upright Olympus BX51WI microscope. Slices were held down with Tungsten/nylon weights and superfused with carbogenated 31°C recording aCSF at 8ml/minute. An inline heater (Harvard) maintained temperature under integrating feedback control by a submerged thermocouple at the bath vacuum port. Although a dual perfusion chamber was not used, modifications were made to improve laminar flow of aCSF over the slice. Bath ground was an Ag/AgCl pellet.

Recordings were performed under visual guidance using near-IR DIC video microscopy using cameras from Hamamatsu and Q-Imaging. Coarse manipulation was performed with 4x magnification, whilst patching was performed under 40x, using micromanipulators from Scientifica.
Custom stimulating electrodes were manufactured using twisted Ni:Cr wire. For TC fibre stimulation, electrodes were inserted into VB thalamus, close to the border with TRn. Biphasic, 100μs constant voltage pulses were TTL-triggered with an optically-isolated stimulus generator (AMPI systems).

Patch recordings were performed with Multiclamp 700B amplifiers (Molecular Devices), using thin-walled borosilicate capillary glass (Warner Precision Instruments). Signals were low-pass (Bessel) filtered at 10KHz and digitised at 20KHz using either a Molecular Devices Digidata 1322A board (for recordings performed in the USA) and pClamp 10.2, or a National Instruments PCI-6110 board (for recordings performed in the UK), using acquisition software custom-written in C++/MATLAB incorporating modules from the freely-available Ephus (http://www.ephus.org) (Suter, 2010).

2.4.1. Cortical area and cell type identification

After positioning the thalamic stimulation electrode, extracellular field potential responses (fEPSPs) were used to determine the position of the strongest connected Layer 4 barrel, guided visually by the barrel wall/hollow contrast in layer 4. Single, low intensity TC stimuli were given at 0.1Hz and <3MΩ patch electrodes, filled with aCSF were used to systematically hunt for fEPSPs. Once barrels had been identified as receiving stable, putatively orthodromic and monosynaptic, clean (amplitude >100μV, onset latency <10ms) fEPSPs, whole-cell recordings were obtained from layer 4 neurons.
Neurons were targeted for patch clamp recordings initially by their somatic morphology under DIC optics, and once whole-cell access was obtained, cell types were confirmed by electrophysiological properties. Excitatory neurons, the principle focus of this study, comprise >80% of neurons in Layer 4 and are predominantly stellate neurons, although no distinction is made between stellate and pyramidal (i.e. with an apical dendrite) neurons in this work. Ex. neurons typically possessed TC inputs that depressed during repetitive stimulation, high (>300MΩ) input resistance, slow membrane time constants (~35ms), and a <100pA rheobase. Additionally, classification distinct to that of non-FS interneurons, the closest cell type both anatomically and electrophysiologically, was possible by spike waveforms in response to depolarising current steps (Daw et al., 2007a; Beierlein et al., 2009).

FS cells were targeted initially under DIC optics as large, elongated somata towards the bottom of the barrel. Initial attempts at targeted interneuron recordings were performed under fluorescence guidance using the GAD67-GFP mouse (Tamamaki et al., 2003), which was crossed to the Fmr1-KO. This approach was eventually aborted (no data shown in this thesis except for example interneurons in Figure 1-3), since deletion of one allele at the GAD67 locus to drive GFP expression leads to a substantial (~50%) reduction in free GABA during development (Y. Yanagawa, personal communication) which has potentially serious effects for comparison against the Fmr1-KO. Nonetheless, experience gained using this mouse led to anatomical criteria adopted for somatic targeting of FS or non-FS cells, such that subsequent electrophysiological identification of FS cells based
upon subjective criteria was as good as a naïve machine classifier given the same parameter set (cluster analysis of layer 4 interneurons is discussed in Appendix 2).

2.4.2. Cell-attached recordings

Cell-attached spike times were recorded as extracellular action currents in voltage-clamp using Cs-based internal solution to subsequently gain whole-cell access. Giga-Ohm seals were obtained, and holding currents that minimised pipette potential against the cell were used (Perkins, 2006). No compensation for liquid junction potential was made, however qualitatively similar results were obtained using pipettes filled with aCSF. Only cells with stable, minimally accommodating >200pA action currents were accepted. High quality (<25MΩ access resistance) whole cell recordings could regularly be obtained following breaking in, even after ~1h in cell attached configuration.

2.4.3. Whole-cell recordings

Whole-cell recordings were made using 4-7MΩ tip resistance microelectrodes. Parasitic pipette capacitance was neutralised using automatic compensation circuitry. Resting potential was measured in bridge balance configuration immediately after breaking in. Criteria for acceptable quality whole-cell recordings included: resting potentials more hyperpolarised than -50mV, stable (<20% drift) access resistance <25MΩ, overshooting APs. Holding currents varied between classes of neurons in Layer 4 as a function of input resistance, but was typically <150pA. Recording quality was monitored on-line by small, hyperpolarising
current/voltage steps and experiments were terminated if any deviation was observed.

In voltage clamp recordings, series resistance was not compensated as only 20-40% compensation could reliably be achieved in layer 4 Ex. neurons - likely due to their small size. Automatic compensation circuitry was found to be unreliable and prone to oscillation during network-evoked current inputs, even in cells in which successful (<80%) series resistance compensation was applied with injected voltage steps. This was particularly obvious in recordings from large FS interneurons. An increase in current noise (due to the lower filtering achieved) was also particularly detrimental in recordings from these cells, which were already substantially noisy due to high rates of spontaneous current input. Correcting series resistance by eye from oscilloscope traces was also avoided due to the subjective bias this introduces, and the potential for compensation error to drift with small changes in access resistance, or pipette capacitance due to slight immersion depth change. Uncompensated series resistance is not anticipated to represent a substantial source of error in voltage clamp recordings from Layer 4 Ex neurons, due to both their high membrane resistance (in comparison to effective access resistance) and the relatively slow and small amplitude currents of interest. If voltage-clamp of Na_v or other fast voltage-gated channel currents was attempted, this would be more problematic (see theoretical discussion in The Axon Guide and Sherman et al., 1999)

In current clamp recordings, uncompensated series resistance introduces more severe distortions relevant to this study. Three adverse effects are
observed: Steady-state voltage errors, time constant distortions, and bandwidth errors.

Firstly, steady-state errors can influence measurements of action potential threshold, since access resistance introduces a systematic voltage error, proportional to injected current amplitudes. For the average wild-type Layer 4 Ex. neuron, with a rheobase current of 80pA, the maximum permitted series resistance of 25MΩ will produce an additional voltage increase of +0.2mV, making the firing threshold appear more depolarised. This problem becomes magnified in lower input resistance cells, such as FS interneurons, where rheobase is ~400pA, and could lead to a spurious 10mV shift.

Secondly, particularly in combination with membrane capacitance ($C_m$), the access resistance of a microelectrode adds an additional parasitic time constant to that of the true membrane time constant, with a constant $\tau \approx R_{\text{series}} \times C_m$. This artificially slows the membrane charging time and can seriously impair the precise recording of both spike times and subthreshold measurements in whole-cell current clamp. This would retard a hypothetical 10-90% step rising time by ~1.8ms in the worst permissible case scenario of 25MΩ series resistance, for the average wild-type Layer 4 Ex. neuron with $C_m=95pA$.

Finally, bandwidth error introduced by series resistance can disproportionately affect high frequency oscillatory potentials, such as those studied in Chapter 3. This effect arises because in combination with

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15 That is to say: $V_{\text{measured}} = V_{\text{membrane}} + (I_m \times R_{\text{series}})$
the membrane capacitance of the cell, the series resistance forms a one-pole RC low pass filter with a -3dB cut-off frequency given by $1/(2\pi R_{\text{series}} \times C_m)$. For the situation presented above, this would restrict bandwidth to 67Hz without series resistance compensation.

To minimise these deleterious effects, series resistance was compensated in current clamp by adjusting bridge balance to minimise instantaneous potential artefacts. Quality of compensation was assessed by examining the second derivative of membrane potential online.

2.4.4. Perforated patch recordings

Gramicidin B was dissolved in DMSO and added to whole cell solution to give a final concentration of 250 $\mu$g/ml. Normal patch pipettes were used. Pipettes were tip filled in normal internal solution before back filling with Gramicidin solution.

Using this approach, gramicidin contained within the patch pipette internal solution gradually diffuses into the patched membrane during the gigaohm-seal configuration, forming anion-impermeable pores in the lipid bilayer that permit voltage-clamp recordings with intact internal [Cl$^-$] (D’Ambrosio, n.d.; Ebihara et al., 1995; Lamsa et al., 2005a). This technique has additionally found favour with experimenters seeking to prevent “wash-out” of intracellular proteins during acute whole-cell plasticity experiments or run down of synaptic release during sustained transmission (Lamsa et al., 2005b, 2007).

Stable perforated recordings were obtained within twenty minutes of obtaining a giga-ohm seal. Access resistance was typically between 25-
60MΩ. Confirmation that whole-cell access had not occurred was obtained by additionally including the fluorophore Alexa-488 in the patch pipette, which rapidly filled the dendritic arbour (visible under epifluorescence) if the patch inadvertently ruptured. Neurons were voltage-clamped and local GABAergic inputs were bulk stimulated with a bipolar electrode approximately 50μm from the soma. In this manner, with all fast glutamatergic and GABA<sub>B</sub> receptors pharmacologically blocked, GABA<sub>A</sub> currents were isolated and could be completely abolished with the further addition of Picotoxin or GABAZine, suggesting that no other neurotransmitter contributed to these responses (Figure 4-1b). GABA<sub>A</sub> current reversal potentials were calculated using linear fits to I-V curves obtained by stimulating inputs whilst sequentially stepping cells between holding potentials of -90mV and -10mV. IV curves were typically linear or weakly inwardly rectifying (Figure 4-1c), when performed in both depolarising and hyperpolarising directions, suggesting that voltage-clamp errors were minimal and that sodium channel inactivation did not substantially improve voltage clamp in the reverse direction. Similar results were obtained using pseudo-random holding potential sequences.

An approach whereby resting potential was estimated extracellularly at the gigaohm-seal stage before the formation of gramicidin pores (Banke and McBain, 2006) was attempted unsuccessfully. This relied upon brief, high amplitude voltage ramps applied across the intact membrane to activate VGKCs. Assuming that the pipette [K<sup>+</sup>] is not drastically different from that
inside the cell\textsuperscript{16}, the cell’s resting potential can be derived from the reversal of the evoked potassium conductance (Verheugen et al., 1999). However, in the present study, perhaps due to low VGKC surface density in Layer 4 excitatory neurons, only weak and inaccurate ramp responses could be obtained using patch pipettes whose size and shape were compatible with subsequent perforated patch formation.

2.5. Recordings extracellular network activity using multi-electrode arrays (MEAs)

2.5.1. MEA recording configuration

MED-P515A (Panasonic MED64) MEA probes were used. These have an 8x8 arrangement of 150\mu m\textsuperscript{2} electrodes, with 150\mu m inter-electrode pitch. Electrodes project 50\mu m vertically into the slice, providing exceptionally low noise (typical impedance: 10k\Omega at 1 kHz) electrodes, even with the fast perfusion used. The raised electrodes have the additional benefit that they improve mechanical stability by increasing surface area in contact with tissue. The “-A” suffix of the probe model number denotes a low profile chamber, rising 5mm above the base glass. This was advantageous, as it permitted the probe to be used nearly interchangeably with the standard recording chamber, with clearance for two patch electrodes, a stimulus electrode, thermocouple, bath ground, solution inflow and efflux, as well as the microscope objective. The integrated MEA amplifier/digitizer was grounded to the Ag/AgCl bath ground, and the 700B patch headstages were left ungrounded – rather, grounded via the 700B amplifier analogue.

\textsuperscript{16} Here, even \sim 10\% error between pipette ([K\textsuperscript{+}]\textsubscript{pip}=155mM) and true internal concentrations introduces minimal Nernst potential error (i.e. RT/F x ln(155/140) <3mV).
ground plane to that of the MEA amplifier. In this configuration, recorded noise of ~5pA R.M.S. and ~10µV R.M.S. were routinely obtained on the patch and MEA channels, respectively. MEA data acquisition was initially performed using proprietary MED64 Conductor software, but later using a custom C++/MATLAB interface that synchronised whole-cell and MEA data sampling via three National Instruments PCI-6110 boards via internal timing.

2.5.2. MEA preparation and use

To reduce surface hydrophobicity, MEAs were prepared for first use by coating overnight at room temperature with a 0.1% solution of polyethyleneimine (PEI, Sigma) in 25mM borate buffer at pH8.4, as per manufacturer’s guidelines\(^\text{17}\). Arrays were then washed 3x with distilled water before use. Between experiments, MEAs were removed from the headstage connector and kept in distilled H\(_2\)O at 4°C.

Slices were positioned on the MEA probe using a paintbrush and were held in place with a slice weight. Care was taken not to scratch the surface insulation or electrodes: each probe costs £180 at the time of writing, and whilst reusable up to 20 times in optimal condition, can be ruined in an instant.

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\(^{17}\) available at http://www.med64.com/resources/methods.html
2.6. Analysis of electrophysiological data

2.6.1. Data processing
All data were analysed using custom-written routines in MATLAB R2009a or R2012a, with the exception of additional toolboxes detailed below. Where necessary to improve analysis speed, C++ or Fortran scripts were compiled and called from within MATLAB as MEX files. For analysis of spontaneous EPSCs and IPSCs, data were imported into Clampfit 10.2, which has an excellent template-based event detection package.

2.6.2. Frequency spectral decomposition
Several experiments detailed in this thesis employ frequency spectral decomposition in signal analysis. Here, I briefly examine methodological trade-offs specific to each application.

2.6.3. Mathematical foundations
Fourier analysis provides the basis for analysis of signals in the frequency domain. Fourier theory holds that an arbitrary periodic function \( f(t) \) with period \( T \) and angular frequency \( \omega \) can be represented by a Fourier series \( P(t) \):

\[
P(t) = \frac{1}{2} a_0 + a_1 \cos(\omega t) + a_2 \cos(2\omega t) + \cdots + a_n \cos(n\omega t) + b_n \sin(n\omega t) + \cdots
\]

\[
= \frac{1}{2} a_0 + \sum_{n=1}^{\infty} [a_n \cos(n\omega t) + b_n \sin(n\omega t)]
\] (Eq. 2-1)
Fourier decomposition seeks to find the $a$ and $b$ terms by minimising the difference between functions $f(t)$ and $P(t)$. Full theory and methodological details are provided in (van Drongelen, 2007, Chapters 5-7).

The Fourier series is often presented in the equivalent complex form:

$$P(t) = \sum_{n=-\infty}^{\infty} c_n e^{in\omega t}, \quad \text{(Eq. 2-2)}$$

with coefficients $c_n$ defined as:

$$c_n = \frac{1}{T} \int_T f(t) e^{in\omega t} \, dt. \quad \text{(Eq. 2-3)}$$

### 2.6.3.1. Discrete Fourier transformation

In order to adapt Fourier analysis for discrete, (i.e. sampled) signals, it is necessary to use finite time and frequency scales. For a signal with duration ($T$) of 1s, recorded at sampling interval ($\Delta t$) of 0.00005s (20kHz) as typically performed in the current work, it is not possible to reliably distinguish below low frequencies to a precision greater than $\Delta f = 1/T$, i.e. 1Hz. Furthermore, the maximum frequency at which a whole period fits within the sampling interval is $1/\Delta t = 1/0.0005s = 20$kHz. In angular frequency notation, these values of precision and maximum frequency correspond to step sizes of $\Delta \omega = 2\pi \times 1/T$ and a range of $\Omega = 2\pi \times 1/\Delta t/2$ rad.s$^{-1}$, respectively, where is $\Delta t$ is further limited by half due to constraints imposed by Nyquist theory.

The discrete Fourier transform $X(k)$, is typically obtained through the computationally efficient Fast Fourier Transform (FFT) approach, yielding:

$$X(k) = \sum_{n=0}^{N-1} x(n) W_N^{kn}. \quad \text{(Eq. 2-4)}$$
To better interpret the complex-valued FFT output, the power spectrum $S$ is usually presented. This plots the power (that is, spectral density) contained at each frequency and is obtained by multiplying the FFT output by its complex conjugate. Representing the FFT output at a given frequency $\omega_k$ as $a_k + ib_k$, the power at $\omega_k$ is:

$$(a_k + ib_k)(a_k + ib_k)^* = (a_k + ib_k)(a_k + ib_k) = a_k^2 + b_k^2,$$  \hspace{1cm} (Eq. 2-5)

where $*$ denotes the complex conjugate, and $i^2 = -1$. This is performed across all frequencies, and the function over the whole spectrum is normalised by the number of data points $N$. The amplitude spectrum $|\tilde{A}|$ is given as the square root of the power spectrum, normalised by $2/N$. Finally, the phase spectrum is calculated from the ratio between the imaginary $I(\tilde{X})$ and real $R(\tilde{X})$ components of the power spectrum:

$$\phi = \arctan \frac{I(\tilde{X})}{R(\tilde{X})}. \hspace{1cm} (Eq. 2-6)$$

2.6.3.2. Spectrotemporal trade-offs and windows

Evaluating the theoretically infinite input signal of the FFT algorithm over a epoch of $N$ samples imposes (i.e. multiplies) a rectangular window on the data. This has the adverse effect that an ideal pure sinusoid in the time domain will be represented not as a single impulse in the frequency domain, but an attenuated main peak flanked by ripples at frequencies equal to the inverse of the duration of the window. In this manner, energy at the fundamental frequency is said to have “leaked”, around the spectral peak. This effect arises from the fact that multiplication in the time domain corresponds to complex convolution in the frequency domain. The distortions imposed by spectral leakage are reduced by the pre-treatment
of data with windows that attenuate side-band ripples. Examples include Gaussian, Bartlett and Hamming windows. Unless otherwise stated, the latter is used in this thesis. No perfect solution exists to this problem (i.e. no finite time domain window can produce a flat line in the frequency domain. Windowing comes at a cost to the height and width of the main spectral peak, essentially reducing broadband bias at the cost of increased narrowband bias - i.e. blurring of signals at distant and nearby signals, respectively. To overcome this in the present study, multi-taper spectral estimates were performed. Here, data are multiplied by not one, but several, orthogonal “Slepian” tapers before taking Fourier spectra. This was implemented using the Chronux toolbox for MATLAB (Bokil et al., 2010). The number of tapers was chosen to best concentrate the spectral properties, as $2NW-1$, where $N$ is the number data points over which the spectra are calculated, and $W$ is the desired analysis half-bandwidth, usually a small multiple of the Raleigh frequency $1/N$. Higher numbers of tapers typically degrade performance.

2.6.3.3. Other approaches: Wavelets/matching pursuit

Whilst Fourier and Hilbert-based spectral decomposition techniques typically decompose signals into functions that are well localised in frequency, they are temporally imprecise (typically smeared ~100ms). This becomes a significant impediment to quantifying short bursts of high frequency activity, typical of high gamma-band activity, or the summed potentials of coherent AP waveforms. These arise as sharp transients, but cannot be represented by these methods using a single function, instead
appearing in spectrograms as nested oscillations across multiple frequencies. Therefore, to minimise the effect of temporal averaging on the analysis of high frequency components associated with spiking activity, a different frequency decomposition approach was taken that permitted higher spectral-temporal resolution (Ray and Maunsell, 2010). Matching Pursuit (MP) is analogous, but considered superior to wavelet decomposition for the study of fast oscillatory components (Mallat and Zhang, 1993; Ray et al., 2008; Bénar et al., 2009). Briefly, in this approach, an overcomplete dictionary of time-frequency elements is constructed, including “Gabor atoms” – Gaussian convolved sinusoids, as well as pure Fourier sines and Dirac δ functions (to provide perfect sinusoids and impulses, respectively). These are then iteratively subtracted from the input signal using a two-step error function until the residual has no further explainable activity. This was implemented in C++/MATLAB using routines kindly made available by Dr. Supratim Ray (http://erl.neuro.jhmi.edu/mpsoft).
2.6.4. Impedance analysis of cortical neurons

2.6.4.1. Calculation of neuronal impedance using sinusoidal current injections

The parallel interaction between membrane resistance and capacitance imparts low pass filter characteristics to the neuronal input-output (current-voltage) transform. The cut-off, or “bandwidth” frequency $f_{\text{bandwidth}}$, of an ideal, first-order low-pass filter, above which the output power relative to DC is attenuated by greater than -3dB (i.e. approximately halved) is given as:

$$f_{\text{bandwidth}} = \frac{1}{2\pi R\text{membrane}} = \frac{1}{2\pi RC}$$  \quad (Eq. 2-7)

Where here $R$ and $C$ are $R_{\text{membrane}}$ and $C_{\text{membrane}}$ respectively. It can therefore be expected that an increase in either $R_{\text{membrane}}$ and $C_{\text{membrane}}$ will, by increasing the membrane time constant, lower the cut-off frequency of the filter effect.

Following previous experimental studies based on now classic techniques (Cole and Curtis 1936, 1938; Carandini et al. 1996, reviewed in Koch 1999), it is possible, by treating the voltage transformation performed upon the current input of a RC circuit as a linear system, to experimentally determine input-output impedance spectra of neurons using discontinuous current clamp recordings.

By injecting a sinusoidal current waveform of single frequency $f$ and amplitude $A$, such that $I_{\text{inject}}(t) = A\sin (2\pi ft)$. According to Fourier

18 with a frequency-dependent roll-off of -20dB/decade
theory, the membrane voltage responds at identical $f$, but with time-shifted phase, such that the membrane potential $V(t)$ follows:

$$V(t) = \tilde{A}(f) \sin (2\pi ft + \phi(f)) \quad \text{(Eq. 2-8)}$$

with a frequency-dependent roll-off in Fourier amplitude $\tilde{A}(f)$ given by:

$$|\tilde{A}(f)| = \frac{R_{\text{membrane}}}{\sqrt{1+(2\pi f \tau_{\text{membrane}})^2}} \lim_{f \to \infty} = 0 \quad \text{(Eq. 2-9)}$$

and frequency-dependent phase shift $\phi(f)$ given by:

$$\phi(f) = -\arctan(2\pi f \tau_{\text{membrane}}) \lim_{f \to \infty} = -\frac{\pi}{2}. \quad \text{(Eq. 2-10)}$$

Finally, the complex valued ratio between Fourier-transformations of voltages (outputs) and current (inputs) can be used to derive the neuron’s input complex impedance $Z(f)$:

$$Z(f) = \frac{\tilde{V}(f)}{\tilde{I}_{\text{Inject}}(f)}. \quad \text{(Eq. 2-11)}$$

The impedance magnitude, $|Z(f)|$ is thus a real valued number between $R_{\text{membrane}}$ for sustained DC current (i.e. Ohmic resistance, $Z(f = 0) = R_{\text{membrane}}$) and zero, decreasing to zero in the limit of $f \to \infty$ as a function of short-circuit through the capacitive reactance (zero for infinitely high frequency). Additionally, the phase shift $\phi(f)$ (in degrees) between the input current and voltage output can be revealed from the inverse tangent of the ratio between the real and imaginary components of the complex impedance:

$$\phi(f) = \angle Z(f) = \arctan \left( \frac{Z(f)_{\text{imaginary}}}{Z(f)_{\text{real}}} \right) \quad \text{(Eq. 2-12)}$$
2.6.4.2. Ideal first-order low pass filter response

First-order (passive) RC low-pass filter responses were assumed as:

\[
Z = \frac{1}{\sqrt{\frac{1}{R_{\text{membrane}}} + i\omega C_{\text{membrane}}}}, \text{ where } \omega = 2\pi f. \quad \text{(Eq. 2-13)}
\]

Impedance magnitude was obtained by taking the absolute value of the complex impedance:

\[
|Z| = \frac{1}{\sqrt{\left(\frac{1}{R_{\text{membrane}}}\right)^2 + \omega^2 C_{\text{membrane}}^2}}. \quad \text{(Eq. 2-14)}
\]
2.7. Analysis of network activity

2.7.1. Analysis of network dynamics from single-cell recordings

2.7.1.1. Spike train statistics

For spike density analysis, spike times from cell-attached recordings were downsampled to 1ms resolution and binned into histograms. Briefly, in this approach each spike time is discretised to 1ms and convolved with a Gaussian probability density function with a Sigma of 15ms. Each spike’s total integral is adjusted to 1 across a range of ±3 Sigma. Correspondingly, spike times were smoothed such that a spike occurs at time t±30ms with 95% certainty, and contributes an extra ~0.1 spike density to that of next spike if it arises 30ms later. The resulting spike density convolution is a continuous distribution rather than a discrete train; more amenable to comparison between trials and recordings, and less sensitive to binning artefacts than with a raw spike count histogram approach.

2.7.1.2. Dynamic Inhibition/Excitation balance

After performing cell-attached recordings of spike times, high quality (R_access < 25MΩ) recordings were obtained using Cs⁺-based internal solution containing QX-314 to improve space clamp and aid the isolation of compound excitatory and inhibitory synaptic inputs. This is particularly important during evoked cortical “UP states” in slice the preparation (Shu et al., 2003), which are reminiscent of high conductance states typical of in
vivo cortical network activity under conditions of slow wave sleep and anaesthesia (reviewed in Destexhe et al. 2003).

Prior to analysis of synaptic currents sustained by feedback activity, raw recordings were smoothed using a Savitzky-Golay FIR filer (polynomial order 3, frame size 15) using the MATLAB function “sgolayfilt”. This was empirically found to remove high frequency stochastic noise whilst maintaining fast synaptic activity, and performed better than typical moving window smoothing filters in this respect.

![Figure 2-1 Savitzky-Golay filtering of recording traces](image)

Raw (left) and S-G filtered (right) whole-cell traces showing improved signal-to-noise ratios. Red circles are detected local minima. Scale: 50ms/50pA.

2.7.2. Analysis of network dynamics from extracellular recordings

2.7.2.1. Data filtering

Data were processed in two parallel streams for Local Field Potential (LFP) and Multiunit Activity (MUA), registered to a common timebase. Raw data were first filtered into LFP and MUA with 5th order digital Butterworth bandpass filters, with pass-bands between 0.1-300Hz and 300-5000Hz,
respectively, designed in MATLAB using the fvtol function. Butterworth filters were chosen for this process as they are maximally flat in the passband, with minimal ripple associated with the transfer function.

2.7.2.2. Estimation of Current-Source Density

2-Dimensional Current-Source Density (CSD) analysis was used to provide spatiotemporal localization of the centre of synaptic activity during propagating network activity.

Following (Nicholson and Freeman, 1975; Nicholson and Llinás, 1975; Łęski et al., 2011), the electric potential Φ is measured at N points, x₁, x₂, …, xₙ. According to Poisson’s equation, the density of current sources, C, and the tissue conductivity, σ, assumed to be isotropic and homogeneous, affect recorded potentials as \( \sigma \Delta \Phi = -C \), where \( \Delta \) is the Laplace operator.

The Laplace operator is typically substituted by the numerical second derivative of extracellular potential, however this assumes that the conduction of extracellular potentials is isotropic, and additionally introduces boundary artifacts at extreme lateral electrode positions.

More recently, inspired by EEG source localization, Łęski and colleagues have developed the application of forward electrostatic modeling, or inverse-CSD (iCSD), which incorporates the known electrode geometries and boundary conditions (Łęski et al., 2011).
Briefly, iCSD analysis assumes a model of current sources $C(x,y,z)$, with parameters $C=[C_1, C_2, \ldots, C_N]$. Every current source then sums in three spatial dimensions to yield the potential at a given point in space:

$$
\Phi(x,y) = \int \frac{C(x',y',z') \, dx' \, dy' \, dz'}{4\pi \sigma \sqrt{(x-x')^2+(y-y')^2+(z-z')^2}}.
$$

(Eq. 2-15)

For 2-D analysis, the $z$ plane is set as zero (i.e. $\Phi(x,y,0)$).

The full set of potentials across each measured point is thus:

$$
\Phi = [\Phi(x_1), \Phi(x_2), \ldots, \Phi(x_N)] = F(C),
$$

(Eq. 2-16)

Finally, the linear operator $F$ is inverted to yield $N$ parameters for the measured potentials:

$$
C = F^{-1} [\Phi].
$$

(Eq. 2-15)

iCSD analysis was performed using a MATLAB toolbox kindly made available by Łęski and colleagues at [http://software.incf.org/](http://software.incf.org/).

Inter-electrode spacing was set at 150$\mu$m. To account for current sources outside the 8x8 grid, the 21 boundary electrodes were extrapolated to give an effective grid size of 10x10. For visual rendering (e.g. Figure 5-14), 2-D CSD maps were 2-D interpolated to 100x100 using cubic splines.

### 2.7.2.3. Spatiotemporal spread of activity

To map the trajectory of propagating current sinks during intracortical network activity, the sink’s centre position at each time-step, $X_{\text{position}}, Y_{\text{position}}$, was acquired from the expression:
\[ X_{\text{position}} = \frac{\sum_{x=1}^{8} CSD_{xy} \cdot x}{\sum_{x=1}^{8} CSD_{xy}}, \quad Y_{\text{position}} = \frac{\sum_{y=1}^{8} CSD_{xy} \cdot y}{\sum_{y=1}^{8} CSD_{xy}}, \]  

(Eq. 2-16)

where \( CSD_{xy} \) is the CSD at time \( t \) recorded from electrode \([x,y]\), and \( x \) and \( y \) are the electrode coordinates on the corresponding eight MEA columns and rows, respectively. The position of the strongest current sink at time each time-point \( t \) was measured and the Pythagorean distance from the position at the start of the analysis window was calculated. Distance traversed and speed of propagation between time-points was calculated after downsampling to 1kHz sampling frequency. Each trial was treated individually, and position vectors were not normalised to starting position or time before averaging across trials. Channels surrounding the sink centre that showed >80% sink density at each time-point, and not radially separated from the sink by an intermediate inactive channel (i.e. one with <80% sink density) were considered simultaneously active at that time-point.

### 2.7.2.4. Multi-Unit Activity analysis

MUA firing rates were estimated by half-wave rectifying the high-pass filtered extracellular signals for each MEA channel and thresholding at eight times the R.M.S. noise in the baseline period. Gaussian kernel-based rate estimates were then computed as for single-cell firing rates, using a kernel with \( \sigma=10\text{ms} \). Estimated firing rates were then compared between trials and slices on the channel that showed the greatest LFP deflection in layer 4.
2.7.2.5. Population vector analysis in principal components.

The trajectories of TC-evoked bursts of firing activity recorded spreading across the MEA were typically variable between trials and slices. Therefore, in order to compare between experiments, Principal Component Analysis (PCA) was applied to the MUA rate estimates. Individual trials were treated independently. MUA rate data were processed at 1kHz downsampled resolution, and activity during the 200ms window of analysis was excised for analysis. Ten trials of 8x8x200ms MUA rate vectors were transformed and concatenated to 64x200x10 and principal components were calculated across the 1st dimension using the princomp function in MATLAB. Geometric mean trajectories for the top three dimensions were then plotted in Figure 5-16, with the width of the plotted vector proportionate to average standard error between trials at each time-point, across the top three dimensions.

2.7.2.6. Spectral coherence

Spectral coherence between two signals, \(x\) and \(y\) is calculated from the cross-spectrum \(S_{xy}\) normalised by the individual power spectra, \(S_{xx}\) and \(S_{yy}\). The magnitude of \(S_{xy}\) is squared to standardise the coherence and provide a dimensionless number between 0 and 1:

\[
C(\omega) = \frac{|S_{xy}(\omega)|^2}{S_{xx}(\omega)S_{yy}(\omega)},
\]

(Eq. 2-17)

with phase coherence given by the ratio:

\[
\Phi(\omega) = \arctan \frac{\text{imag}(S_{xy}(\omega))}{\text{real}(S_{xy}(\omega))}.
\]

(Eq. 2-18)
Spectral coherence was calculated using routines from the Chronux toolbox. Coherence was investigated between the strongest Layer 4 channel, and surrounding co-active channels, defined by >80% maximum sink density as above. LFP-LFP coherence was calculated using the coherency function, with a time-bandwidth product of 3, and 5 Slepian tapers. LFP-MUA coherence was calculated using the coherency function, using MUA rates binned at a temporal resolution of 1kHz, with a time-bandwidth product of 10, and 4 Slepian tapers.

2.8. Computational modelling: neuronal synapses and circuits

Computational modelling was performed in the NEURON simulation environment (Hines and Carnevale, 1997). Simulations were run with multi-order time step integration, which was found to speed up simulations by a factor of ten compared to standard fixed time step integration. NEURON 7.2 was compiled from source under Ubuntu 12.04, running on a 64-bit AMD64 architecture machine (quad-core 2.5GHz Intel Core i7, 8GB RAM).

The general workflow for TC synaptic integration simulations performed in Chapter 4 was to load a standard model neuron, then apply combinations synaptic and intrinsic parameters as measured in wild-type and Fmr1-KO neurons. Two nested loops then provided model synaptic stimulation at varying inter-stimulus intervals and strengths of FFI. For each iteration in the loop, the maximum depolarisation evoked by model
inputs was measured by the simulated membrane potential response, and the values were saved to a vector for later analysis in MATLAB.

2.8.1. Synaptic transmission

The model synapses used in this study are based on phenomenological descriptions of short-term plasticity at synapses (Varela et al., 1997). Substantially more detailed models of short-term synaptic dynamics are available, but require more parameters, such as Ca\(^{2+}\) buffering and accumulation to be measured before simulations can be reliably constrained. In the “FDSExp2Syn” model used, the synaptic mechanism itself is described by a two state kinetic scheme, described by rise time \(\tau_1\) and decay time constant \(\tau_2\). The normalised peak conductance is 1.

The solution for the state transition \(A->G->\text{bath}\) with rate constants \(1/\tau_1\) and \(1/\tau_2\) for synaptic rise and decay times, respectively is given by:

\[
A = a * e^{-t/\tau_1}, \quad \text{and} \quad (Eq. 2-19)
\]

\[
G = a * \frac{\tau_2}{\tau_2 - \tau_1} * (e^{-t/\tau_1} + e^{-t/\tau_2}),
\]

where \(\tau_2 > \tau_1\).

To model short-term depression of TC-EPSCs and FF-IPSCs, which typically depressed in physiological recordings with at least one exponential term, the following dynamic response scheme was adopted from Varela et al. (1997). For each current component independently, starting from steady-state amplitude \(A_0\), the stimulus evoked response
amplitude $A$ was multiplied by two dynamic depression factors, $D_1$ and $D_2$ (constrained <1):

$$A = A_0 D_1 D_2.$$  \hspace{1cm} (Eq. 2-20)

After each stimulus, $D_1$ and $D_2$ were multiplied by constants $d_1$ and $d_2$, representing the amount of depression per presynaptic action potential (i.e. $D_1 \rightarrow D_1 d_1$, and $D_2 \rightarrow D_2 d_2$). Between stimuli, $D$ variables recovered exponentially back towards 1 with first-order kinetics governed by recovery time constants $\tau_{D1}$ and $\tau_{D2}$:

$$\tau_{D1} \frac{dD_1}{dt} = 1 - D_1, \text{ and } \tau_{D2} \frac{dD_2}{dt} = 1 - D_2.$$  \hspace{1cm} (Eq. 2-21)

Accumulation of synaptic depression was therefore observed when the inter-stimulus interval was less than that required for recovery.

Rise and decay time constants $tau1$ and $tau2$, as well as synaptic plasticity constants $d_1$, $d_2$, $\tau_{D1}$ and $\tau_{D2}$ were obtained from the mean synaptic kinetics and short-term depression of EPSCs and FF-IPSCs individually. Fitting of short-term depression terms was performed on mean normalised synaptic depression curves for 5, 10, 20 and 50Hz TC stimulation, optimised using a Levenberg-Marquardt search implemented in MATLAB.

### 2.8.2. Compartmental models

To approximate the passive properties of a Layer 4 Ex. neuron somatic patch clamp recording, a “ball and stick” morphology was adopted. A single compartment soma (10µm diameter and length) was attached to a length of dendrite (50 compartments, <1mm long, 3µm diameter). The
global leak conductance and "dendritic" length was adjusted differentially for the wild-type and *Fmr1-KO* models such that the mode input resistance, capacitance and emergent time constant matched those of genotype mean values. Leak reversal potentials were -60mV, to approximately match the resting potentials of recorded neurons.

Two FDSExp2Syn model synapses were added to the soma, with synaptic properties tuned to genotypes as discussed above. To approximate the TC AMPA component, one was given a fixed peak conductance and reversal potential of 0mV such that it evoked ~100pA inward current when the model was “clamped” at the reversal potential of the model IPSC current. The model GABAergic FF-IPSC current had a reversal potential of -71mV corresponding to the chloride reversal potential in physiological recordings as set by the internal solution. The range of peak FF-IPSC conductances that evoked preset FFI ratios was determined empirically by “voltage clamping” the model soma at 0mV and activating the model IPSC. Both EPSC and IPSC synapses were connected to the same Netstim block in the NEURON simulation, such that stimulation times were shared. However the disynaptic FFI delay was implemented by staggered activation delays in the model current onsets. Similarly, as discussed above, the short-term depression of model EPSCs and FF-IPSCs was stream specific, such that depression of model FFI was faster that that of model TC inputs.
2.9. Statistical tests

Unless otherwise specified, all statistical tests are parametric and performed using Student’s t-test. Prior to each statistical comparison, normality of each dataset was tested with both Komogorov-Smirnov and Shapiro-Wilk tests for normality. If either or both test groups were found not to be normally distributed, non-parametric tests, typically Mann-Witney, were used. Central tendency is reported for all data as Mean±SEM. Where appropriate, Median±range are also quoted. All statistical tests are two-way, with significance level quoted at 95%. To avoid misrepresenting potentially pseudoreplicated data, numbers of data-points, where not explicitly quoted as number of slices and animals used, are assumed to include multiple cells per animal.
3. Loss of FMRP distorts intrinsic neuronal electrophysiology and circuit connectivity in the P10/11 cortical Layer 4

3.3. Key findings:

1. In young *Fmr1* KO neocortical layer 4, excitatory neurons show elevated intrinsic excitability and slower membrane time constants which distort their sub- and supra-threshold responses to a variety of current stimuli; namely forced depolarization, sinusoidal waveforms and spontaneous synaptic input.

   a. Frequency-dependent processing of oscillatory stimuli is affected in these neurons, enhancing sensitivity to low (delta-theta band) input, whilst proportionally attenuating sensitivity to higher (gamma band) frequencies.

   b. These effects are demonstrated at holding potentials ±10mV of resting potential, and conform to a simple RC model circuit, together consistent with the involvement of elevated passive membrane resistance.

   c. These effects are not consistent with a simple developmental delay in the maturation of cell-intrinsic properties.

2. Fast-Spiking interneurons - a predominant class of GABAergic cell in layer 4 - show similarly altered intrinsic properties in young *Fmr1*-KOs, with changes observed in action-potential shape and input resistance.

3. Synaptic connectivity between principal excitatory neurons and Fast-Spiking interneurons, which provide powerful basket inhibition, is dramatically (~50%) and bidirectionally reduced in the absence of FMRP.

4. Cell-type specific bidirectional changes in the strength and frequency of spontaneous synaptic input, a marker of synaptic connectivity, are observed in layer 4.
3.4. Introduction

In this first data chapter I explore how abnormal neuronal intrinsic properties and synaptic connectivity of two predominant classes of neurons in Layer 4 of the juvenile Fmr1-KO barrel cortex distort neuronal input-output transformations under controlled experimental conditions. To ask whether maturation of the mutant neurons is developmentally delayed, I explore the normal development of Layer 4 neurons’ intrinsic physiology during the second postnatal week of life. I also discuss implications for processing of sensory input and provide firm grounding for later chapters testing TC-evoked circuit activity.

3.5. Results

3.5.1. Elevated intrinsic excitability of young Fmr1-KO neurons

To first assess how the intrinsic current-voltage transformations performed by Layer 4 neurons are affected in the absence of FMRP, responses to injected current inputs were evaluated under controlled conditions. Patch clamp recordings were made from Layer 4 excitatory neurons in brain slices. Neurons were maintained at -70mV in current clamp configuration, close to resting membrane potential\(^\text{19}\). Putative excitatory neurons were visually targeted by somatic morphology and laminar position under DIC optics, and identity was confirmed in whole-cell configuration by AP firing pattern in response to stepwise increases in injected current. Figure 3-1a shows exemplar firing responses to rheobase (coloured) and twice-

\(^{19}\) Resting membrane potential was not significantly different between genotypes – see b
rheobase current injections for excitatory neurons in slices from P10-11 Fmr1-KO animals (red, labelled Fmr1<sup>+/Y</sup>) and wildtype (blue, labelled Fmr1<sup>+/Y</sup>) littermates. This colour convention will be maintained throughout this thesis. Fmr1<sup>+/Y</sup> and Fmr1<sup>+/Y</sup> notation is used in the figures to denote hemizygous male animals, whereas Fmr1-KO and wild-type nomenclature are maintained in the text for clarity without resorting to colour labels.

Several changes were observed in the firing patterns of Fmr1-KO neurons in response to forced depolarisation. 500ms depolarizing current pulses from -70mV holding potential could elicit a single AP with a significantly lower rheobase current in Fmr1-KO neurons compared to those from littermates (Figure 3-1c). Successive suprathreshold 10pA increases in amplitude elicited repetitive firing trains with significantly more APs in Fmr1-KO neurons: F-I slopes for the linear region, i.e. firing rate gain in response to increased current injection, measured in Hz/nA, were significantly steeper. Together, these results suggest a F-I curve manipulation that contains both additive offset (rheobase) shift and multiplicative gain operations in Fmr1-KO neurons relative to those from wild-type littermates.
**Figure 3-1** Spiking behaviour of layer 4 excitatory neurons under *ex vivo* conditions

**a).** Example voltage responses of L4 excitatory neurons to injected current steps (500ms duration) in whole-cell configuration. Black traces indicate response to -40pA and rheobase (threshold, eliciting one action potential) current. Coloured traces indicate responses to twice-rheobase current. Note the lower rheobase current and shorter actions potentials for the example -Y neuron.

**b).** Cyclic phase plots for the twice-rheobase example responses shown above. Note slower action potential rising phase (maxima on ordinate).

**c).** Left: Threshold current (rheobase) required to evoke one action potential in a Layer 4 excitatory neuron. Asterisk indicates p<0.05, t-test. Symbols indicate individual neurons. Right: number of action potentials evoked by each current injection, in 10pA increments. Linear fits to F-I slopes were significantly different between genotypes (120±10Hz/nA (*Fmr1*+/Y) and 200±12Hz/nA (*Fmr1*−/Y), Mean±SEM, (p<0.05, t-test). Asterisks indicates p<0.05, individual t-tests for results obtained at each current step, N= 28 neurons for each genotype.
3.5.2. Action Potential waveforms are altered in young *Fmr1-KO* neurons

AP waveform shape is critically linked, via presynaptic calcium influx and presynaptic release, to the efficacy of synaptic transmission (Bean, 2007). Recent studies implicate loss of FMRP via altered AP shape to dysregulated synaptic release (Rotman et al., 2011; Deng et al., 2013). In the present study, changes in the spike shape were observed that suggested slower AP dynamics in neurons from *Fmr1-KO* slices. Figure 3-1b presents alternatively the repetitive AP firing at twice-rheobase current injection of cells in shown in Figure 3-1a. Here, cyclic phase plots show the first derivative of membrane potential as a function of membrane potential to highlight relaxation of AP shape during sustained firing. Note both the slower AP rise and fall speeds (maximum and minimum on abscissa, respectively) for the first AP in the train (exterior cycle in plot), and the progressive reduction in speed for successive APs (cyclic decay of maxima).

To quantify dynamic changes in spike shape during repetitive firing, an analytical approach was adopted whereby AP waveforms were automatically annotated, identifying waveform features including AP peak height and width, rise and fall speed and threshold²⁰ (Figure 3-2a). Dynamic changes to AP shape parameters were compared between genotypes as a function of AP number in the evoked train (Figure 3-2b). AP rising and falling speeds were significantly slower in *Fmr1-KO* neurons on the first AP and although significantly greater relaxation in AP rise speed

---

²⁰ AP voltage threshold was designated as the point at which voltage acceleration during the AP rising phase increased above 10mV/ms.
was observed on the second AP in *Fmr1-KO* neurons, differences between genotypes for subsequent APs were largely consistently maintained throughout the duration of the 500ms response. AP half-widths were initially wider in *Fmr1-KO* neurons. Since AP duration is largely controlled by voltage-gated K+ conductance type and density (Bean, 2007), a strong candidate underlying the broader spikes shown here is an alteration in expression of VGKCs. Indeed, Deng and colleagues recently demonstrated a similar spike broadening that was attributable to abnormal BK channel activity in the absence of FMRP (Deng et al., 2013).

In *Fmr1-KO* neurons, AP heights (measured above threshold) were not initially significantly different to those from wild-type neurons but showed significantly stronger amplitude attenuation during repetitive firing. This effect is consistent with the effect of a strong progressive reduction in peak suprathreshold *Na*<sub>V</sub> conductance density (Milescu et al., 2010). This could arise either from lower channel density, or more progressive channel inactivation during repetitive firing, however the impact of these parameters is modulated additionally by the passive membrane time constant. The overall effect of slower depolarisation speed would limit peak AP height, and additionally reduce activation of voltage-gated potassium channels (VGKCs), limiting in turn the rate of repolarisation and broadening the spike width. It is noted however that in addition to VGKCs, subthreshold *Na*<sub>V</sub> conductance density can contribute to AP pacemaking activity and firing rate (Taddese and Bean, 2002; Milescu et al., 2010).

Mean AP threshold was significantly depolarised in *Fmr1-KO* neurons on the first AP (Figure 3-2b) and maintained in this state for the duration of
the response train. This effect could be a secondary effect of gradual ionic accumulation during repetitive firing, however the genotype-specific difference was present on the first AP. Since both the mean and first derivative of the membrane potential have been shown to affect spike threshold (Azouz and Gray, 2000, 2003), and membrane potential derivatives were significantly slower in $Fmr1$-KO neurons (Figure 3-2b), this latter effect is more likely to be a causative factor. This interpretation is consistent with the observation that changes to membrane potential derivatives are involved since AP width and threshold were largely consistent during the AP train rather than showing independent accommodation.

Finally, firing rate was also significantly slower in $Fmr1$-KO neurons: instantaneous AP frequencies, measured during twice-rheobase current injections to normalize for changes in membrane resistance ($R_{\text{input}}$), were significantly lower for the first four APs in the train (Figure 3-2b, bottom right, however frequency relaxation took place at the same rate for each genotype.

Overall, some parameters of excitatory neuron firing (AP falling speed, half-width and firing rate) were significantly different between genotypes on the first AP, appearing stably different throughout evoked AP trains and suggesting that AP broadening did not take place as a function of AP number. Conversely, changes in AP amplitude accommodation emerged as a function of AP number.
Disentangling the contributions of individual ionic conductances to these phenotypes requires further experimentation. To this end, in companion to pharmacological isolation, an attractive approach would be to take advantage of fast dynamic clamp techniques. Here, one can evaluate state-based kinetic models of channel activity calculated in real-time and injected into a neuron in which the real biological channels have been rendered inactive with blockers (Milescu et al., 2008). This approach could be advantageous in disease model neurons to simultaneously examine the biophysical fine details (e.g. inactivation kinetics – requiring time-consuming experiments to obtain using traditional methods) of defective conductances, thereby rapidly building and testing hypotheses to best return healthy function to a dynamic system in which multiple parameters change in parallel – without making assumptions a priori about causative or secondary effects of channel density changes.
Figure 3-2 Exaggerated dynamic action potential shape changes during repetitive firing of $Fmr1^{+/Y}$ neurons in response to 2x rheobase current injection.

a). Example analysis demonstrating automatic action potential annotation algorithm processing an $Fmr1^{+/Y}$ neuron. Upper: raw voltage trace, Lower: first voltage differential with respect to time.

b). Action potential shape changes compared between $Fmr1^{+/Y}$ (blue) and $Fmr1^{-/Y}$ (red). For each parameter, measured values (top) are additionally shown as normalised to the value on the first AP (bottom). Asterisks indicate significantly different parameters spike $(p<0.05$, t-tests comparing values for each spike, N: $Fmr1^{+/Y}=50$ neurons, $Fmr1^{-/Y}=42$ neurons)
I inject (2x rheobase current) $I_{\text{inject}}$

**Maximum rise slope**

**Maximum fall slope**

**AP peak**

**AP threshold**

**AHP trough**

**Depolarization speed**

$\text{max } dV/dt (\text{mV.mS}^{-1})$

**Repolarization speed**

$\text{min } dV/dT (\text{mV.mS}^{-1})$

**AP Amplitude (mV)**

**AP threshold (mV)**

**Instantaneous frequency (Hz)**

**AP Half-Width (ms)**

**AP number**

*Denotes significant differences.*
3.5.3. Elevated membrane resistance distorts membrane time constants of *Fmr1-KO* Layer 4 excitatory neurons

To what extent do the changes to intrinsic AP-firing behaviour in *Fmr1-KO* neurons emerge as a function of altered intrinsic membrane properties? An elevated input resistance ($R_m$) could be expected to contribute to the subtractive shift of rheobase current observed in *Fmr1-KO* F-I slopes presented in Figure 3-1c and could also affect the effective membrane time constant ($\tau_m$) (Azouz, 2005; Silver, 2010), which could alternatively be obtained through increased membrane capacitance ($C_m$). To obtain an experimental measure of membrane resistance and capacitance, voltage deflections in response to small amplitude, hyperpolarising current steps were recorded from current-clamped neurons maintained at -70mV, close to resting potential (Figure 3-3a). Since this approach minimises contributions from voltage- or hyperpolarisation-gated currents, it is reasonable to assume that only linear (Ohmic) input resistance is obtained by this manipulation. Additionally, only cells with electrical access resistance of better than 25MΩ were included for analysis, and space clamp is of good quality due to the compact electrotonic properties of Layer 4 excitatory neurons. Furthermore, minimal sag current – transient $I_h$ current and a potential source of experimental error - was observed in these recordings. Thus, experimental artefact is not expected to significantly contaminate these measurements.
Figure 3-3 Passive properties of P10-11 Fmr1+/Y and Fmr1−/Y L4 excitatory neurons.

(a). Example data showing procedure for calculating passive properties of neurons from current clamp data. Neurons were maintained at -60mV, close to rest. Small hyperpolarising current steps were applied to produce negative membrane potential deflections without evoking active currents (e.g. $I_h$). Thick dotted lines indicate single exponential fits used to derive membrane time constants ($\tau_{membrane}$).

(b). Intrinsic membrane properties of excitatory neurons. Asterisks indicate p<0.05, t-test. Data shows mean±SEM. Symbols indicate individual neurons.

Strikingly, input resistance, as measured from steady-state voltage deflections was significantly elevated by approximately 60% in Fmr1-KO neurons compared to those in slices prepared from littermate mice (Figure 3-3a,b). To estimate the effective membrane time constant of these neurons, onset kinetics of the recorded voltage deflection slopes were fit with an exponential decay function and conformed well to single exponential terms, again suggesting that little deviation from a passive (i.e. $R_mC_m$ circuit) response was obtained, and that good space clamp and a contiguous charging of dendritic capacitance was obtained.
Similar to the change observed in input resistance, membrane time constants were significantly longer in Fmr1-KO neurons by approximately 60%. Correspondingly, no change was observed in effective whole-cell capacitance, as derived from \( \tau_m = R_m \times C_m \), suggesting that total membrane surface area (as a proxy for dendritic arbour elaboration) or membrane lipid composition were unaltered in mutant neurons.\(^{21}\) Resting membrane potential, measured within ten seconds after gaining whole-cell access in voltage-follow configuration (i.e. without applying bias current), was also not significantly different between neurons of either genotype, indicating that neurons were similarly healthy and electrogenic in both cases.

### 3.5.4. Intrinsic neuronal properties of Fmr1-KO Layer 4 excitatory neurons with reference to normal developmental trajectory

In order to examine whether alterations to the firing properties of Fmr1-KO neurons found in the present study occurred as a result of a delay in the developmental maturation of their intrinsic properties, recordings from P10-11 mutant neurons were additionally compared to those from younger wild-type neurons. A developmental series between P6-P11 depicting changes to spike waveforms in response to rheobase current is shown in Figure 3-4.

\(^{21}\) This is corroborated by anatomical data showing unchanged dendritic branching in Till et al. (2012).
Figure 3-4 Properties of Fmr1^{-Y} (red) excitatory neuron action potential shape at P10-P11 with reference to the developmental progression of wildtype (Fmr1^{+Y}, blue) action potentials from P6-P11.

Action potential shapes were measured from spikes from rheobase traces (i.e. in response to minimal current injection producing a single action potential). Kruskal-Wallis test, Dunn’s multiple comparison test, data points indicate individual neurons.

Asterisks indicate significant comparisons (at p<0.05 confidence level) with the following scheme based upon mean rank separation: * >20, ** >30, *** >40.
The developmental changes observed are summarised as follows: The shape of the after-hyperpolarisation (AHP) did not change significantly over this period. However, significant developmental trends were observed in AP height, half-width, and peak rise and fall speeds, becoming taller, narrower and faster respectively. Of these trends, a subtly weaker developmental separation was observed for AP half-width and AP height between P6-7 wild-type and P10-11 Fmr1-KO neurons relative to the same developmental comparison for P10-11 wild-type neurons, and between P8-9 and P10-11 for AP rise and fall speeds.

A similar comparison was made for developmental changes to passive membrane and general excitability properties and firing properties of the same neurons (Figure 3-5, upper). A general trend towards lower input resistance was observed in wild-type neurons between P6-7 and P10-11 that was only significantly different to P10-11 Fmr1-KO neurons when compared to age-matched P10-11 recordings. Membrane time constant yielded the same results in this developmental comparison. Additionally, while mean membrane capacitance increased slightly between P6-7 and P8-9, little change was observed past this age.

General excitability (firing threshold, rheobase and firing rate at twice-rheobase) was also examined (Figure 3-5 lower). AP threshold showed a similarly paced but opposite developmental trend to changes observed in AP amplitude. Despite an increasing trend, rheobase did not become significantly different between developing wild-type neurons across the ages studied.
**Figure 3-5** Intrinsic membrane properties of Fmr1<sup>−/−</sup> (red) excitatory neuron at P10-P11 with reference to the developmental progression of wildtype (Fmr1<sup>+/−</sup>, blue) neurons from P6-P11.

Kruskal-Wallis test. Dunn's multiple comparison test, data points indicate individual neurons. Asterisks indicate significant comparisons (at p<0.05 confidence level) with the following scheme based upon mean rank separation: * >20, ** >30, *** >40.
Mean rheobase was significantly lower in P10-11 Fmr1-KOs than for any other wild-type age range except P6-7, thus emerging as a developmental deficit more severe than that of the underlying increase in input resistance. Finally, a general but highly scattered developmental trend towards fewer spikes fired at twice-rheobase current injection was observed for wild-type neurons. Comparison between time-points for this dataset and P10-11 Fmr1-KO neurons suggested the lowest mean value was for the latter, (only significantly different when compared to P8-9 wild-type neurons) despite not being significantly different between age-matched wild-type neurons (p=0.064, P10-11 wild-type vs. P10-11 Fmr1-KO, Mann-Whitney).

3.5.5. Impedance spectral characterisation of Fmr1-KO excitatory neurons reveals altered impedance

The 60% increase in input resistance measured in Fmr1-KO excitatory neurons reported above is predicted to drastically alter the performance of these cells when responding to oscillatory current inputs. Indeed, predicted values of $f_{\text{bandwidth}}$, for low-pass filter properties of wild-type and Fmr1-KO neurons as calculated from the genotype means of $R_{\text{membrane}}$ and $C_{\text{membrane}}$ in the present P10-11 dataset are 4.12Hz and 2.94Hz, respectively. Strong (20dB/decade) frequency-dependent attenuation is expected for frequencies outwith this pass-band.

These predictions were experimentally tested in Layer 4 excitatory neurons by calculating impedance spectra using Fourier analysis (See chapter 2 – Materials and Methods). An example of this approach is illustrated in Figure 3-6a.
Figure 3-6 Procedure for experimentally measuring intrinsic impedance spectra of neurons by patch-clamp electrophysiology (single trial example shown).

a). A sinusoidal forcing function (“ZAP”) with constant amplitude, monotonously increasing frequency sweep (“I_inject”, orange ramp) is applied to current-clamped neuron via patch pipette. Resulting membrane potential deflections (“V_m”) are recorded. Note frequency-dependent amplitude attenuation of membrane potential. b). Example steps in reconstructing impedance profile. Top: single-sided amplitude spectrum (FFT, Hamming-windowed) of input current sweep. Note spectral leakage. Middle: single-sided amplitude spectrum (FFT, Hamming-windowed) of membrane potential. Bottom: Impedance magnitude (absolute ratio of FFT(V_m) to FFT(I_inject)).
Current-clamped neurons were maintained at a holding potential of -65mV and subjected to a constant-amplitude rising frequency sweep (“ZAP”) that increased linearly between 0.5Hz and 50Hz over 25 seconds. To standardise between changes in input resistance of neurons, injected current amplitudes were scaled to produce a +10mV deflection in membrane potential during a 10 second 0.5Hz sinusoid. Current injection and voltage response recordings were then Hamming-tapered and decomposed into Fourier series using the Fast Fourier Transform. Note the flat Fourier amplitude reconstructed from $I_{inject}$ in Figure 3-6b despite spectral leakage. This example neuron had a DC input resistance of 580MΩ, measured at -70mV as described previously. Note the asymptotic convergence of experimentally derived input impedance towards this D.C. value at low frequencies, demonstrating that minimal response attenuation occurred at frequencies below 1Hz.

This process was performed for wild-type and Fmr1-KO neurons - example responses to ZAPs 0.5-50Hz are shown in Figure 3-7a. Stronger frequency-dependent amplitude attenuation relative to that for 0.5Hz was observed in Fmr1-KO neurons relative to those from littermates. Population averaged mean±SEM impedance spectra for six Layer 4 excitatory neurons (one cell per animal) of each genotype are shown in Figure 3-7b (left). In agreement with the increased D.C. input resistance reported above, impedance to low frequency oscillatory current stimuli was strikingly increased in Fmr1-KO excitatory neurons, and was significantly elevated for frequencies between 0.5Hz and 4Hz.
**Figure 3-7** Altered impedance spectra of *Fmr1-KO* excitatory neurons

**a).** Example responses to 20s frequency sweeps highlighting response for 4 cycles following $f=0.5\text{Hz}$, $f=10\text{Hz}$ and $f=20\text{Hz}$. Black traces: input current, peak-peak amplitude is shown scaled to 1. Coloured traces: voltage responses, scaled to maximum depolarising amplitude at low frequency end of sweep, approx. $+10\text{mV}$ from holding potential. Note progressive attenuation in amplitude and phase shift visible in inset.

**b).** Left: Population average impedance spectra for tested excitatory neurons. Plots show mean±SEM responses. Grey shaded region indicated frequencies showing significantly different ($p<0.05$) impedance magnitudes, between $0.5\text{Hz} - 4\text{Hz}$. Statistical significance was evaluated by performing t-tests between data binned in 0.5Hz increments. N=6 neurons for each genotype, one per animal. Right: Simulated impedance profiles for (passive) model RC circuits matching genotype mean input resistance and membrane capacitance measurements (see Figure 3-3). Shaded region highlights frequencies significantly different between genotypes in experimental data (on left).
Consistent with the absence of a significant inductive or resonant components (see discussion in this section: 3.4.2), empirically derived spectra were in good accordance with ideal impedance spectra for first-order low-pass filters modelled from genotype mean $R_{\text{membrane}}$ and $C_{\text{membrane}}$ (Figure 3-7b).

It is conceivable that both spectral leakage and under-representation of low frequencies during Fourier analysis and the presence of small amounts of (non-linear) $I_h$ in experimental data contribute to discrepancies between the model and data spectra. Similar findings were obtained by using ZAPs with different ramp speed, and compound (ramp up, ramp down) ZAPs (data not shown). Moreover, injecting 10s of discrete, constant frequency waveforms into excitatory neurons led to similar findings (Figure 3-8), and the range of frequencies over which impedance differed significantly between genotypes was extended from 0.5Hz up to 7Hz. This increased range is likely due to inter-cell variation in intrinsic properties between the two datasets.
Impedance magnitude spectra of L4 excitatory neurons as estimated using 10s duration, constant amplitude current injections with static frequency.

Responses show average of 3 trials/frequency/neuron. Current amplitudes were maintained constant for successive frequencies for individual neurons and were determined as for Figure 3-7. Plots show mean±SEM responses. Grey shaded region indicated frequencies showing significantly different (p<0.05) impedance magnitudes, between 0.5Hz – 7Hz. Statistical significance was evaluated by performing individual t-tests for each frequency at 0.5Hz increments. N: Fmr1+/Y = 10, Fmr1-/Y = 7 neurons (different cells from those analysed in Figure 3-7).
3.5.6. Distortions to frequency filtering and following behaviour by *Fmr1-KO* Layer 4 excitatory neurons

What are the physiological consequences of the elevated impedance to low frequencies in *Fmr1-KOs*? Treating the neuronal current-voltage response transformation as a linear system, one can take advantage of standard engineering notation to analyse its input-output characteristics. Figure 3-9 re-evaluates the data presented in Figure 3-7 as Bode gain and Bode phase plots and highlights changes to low-pass filter responses of excitatory neurons between the two genotypes.

3.5.6.1. Changes to gain current-voltage gain in *Fmr1-KOs*

In Figure 3-9a, the frequency-dependent system gain is expressed as log-power ratios of impedance at higher frequencies relative to that at the lowest tested (f=0.5Hz). Little additional output attenuation compared to DC is expected for either genotype at 0.5Hz, i.e. where $R_{\text{membrane}} \approx Z_{(f=0.5Hz)}$, hence this is a fair normalisation strategy (Zemankovics et al., 2011). This plot illustrates two predictions from model responses (annotated in Figure 3-9 on the left-hand panels). Firstly, power attenuation to below -3dB (i.e. the point at which power is approximately halved relative to that at f=0.5Hz, or, where magnitude drops below $\frac{1}{\sqrt{2}} \cdot Z_{0.5Hz}$, defined as $\frac{1}{2\pi RC}$ for a passive linear RC circuit) is predicted to be lower for *Fmr1-KO* neurons relative to those from littermates – excessively restricting the “pass-band”, the range of input frequencies that experience minimal output attenuation relative to DC.
**Figure 3-9** Low pass filter characteristics of L4 excitatory neurons are altered in Fmr1-KO neurons.

Comparison between data from sinusoidal sweep experiments presented in Figure 3-7 and simulated ideal (i.e. passive one-pole) RC low-pass filter estimated from the mean $C_{\text{membrane}}$ and $R_{\text{input}}$ of each genotype.

**a).** Bode filter analysis for simulated (left) and experimental (right) responses. One-pole 20dB/decade response power roll-off and approximate half-power reduction (“cut-off frequency”, -3dB) are indicated with dotted lines. Mean $F_{\text{cut-off}}$ was significantly reduced in Fmr1$^{-/Y}$ neurons by approx. 3Hz (t-test, n=6 neurons for each genotype). Plotted experiment data indicates mean±SEM response.

**b).** Simulated (left) and experimentally measured (right) current-voltage phase distortions calculated empirically (model) by Fourier analysis (data). Plotted experiment data indicates mean±SEM responses. Average phase shifts between 1-10Hz were significantly greater for Fmr1$^{-/Y}$ compared to Fmr1$^{+/Y}$ neurons (t-test, n=6 neurons for each genotype).
Secondly, the power roll-off, or slope of attenuation in the stop-band, is expected to be -20dB/decade for both genotypes, such that extent of attenuation within the stop-band will be equal regardless of the ratio of frequencies examined. These predictions were well borne out by the data, and a significant reduction was observed in mean cut-off frequency between genotypes (Fmr1+/Y vs. Fmr1-KO, model: 4.12Hz vs. 2.94Hz; data: 4.65±0.45Hz vs. 1.89±0.23Hz, mean±SEM).

3.5.6.2. Changes to current-voltage phase shift in Fmr1-KOs

For the genotype mean passive model RC circuits, the effect of input frequency on input-output phase shift is shown in Figure 3-9b. The phase shifting effect of an ideal RC low-pass filter circuit can be conceptually summarised as follows: When DC or very low frequency input current flows, the membrane capacitance is effectively an open circuit, thus all current flows Ohmically through the membrane resistance, with no shift in output phase. With input current of infinitely high frequency however, short-circuit introduced by the drop in capacitive reactance draws all injected current, and according to Kirchhoff’s first law, there is an infinitely high voltage drop across the resistor such that $I_R=0$. Here, the capacitor current phase matches that of the input current. However, since the voltage drop across the capacitor $V_C = \int I_C(t)/C$, and since $\int \cos(x) = -\sin(x)$, the output voltage is a copy of the input but shifted -90 degrees in phase. For intermediate frequencies, the shift in voltage phase interpolates monotonically between the two extremes with phase angle $\phi = -\arctan(i\pi f \cdot R_{membrane} C_{membrane})$ (as shown in Figure 3-9b (left)).
reflecting the relative current flow between the membrane resistance and capacitance, dictated by the capacitive reactance at that frequency.

Subsequently, since $R_{\text{membrane}}$ was elevated in recordings from $Fmr1$-KO neurons, the rate at which the voltage phase shift progresses from 0 towards -90 degrees with increasing input frequency is expected to be faster in these neurons, that is, for a given frequency, a greater phase shift is expected in $Fmr1$-KO neurons compared to those from wild-type animals. This was confirmed experimentally, as shown in Figure 3-9b (right). Current-voltage phase shifts were significantly greater in $Fmr1$-KO neurons compared to those from wild-types by an additional 10 degrees as averaged between 1 and 10Hz.

These and the previous experiments were performed by biasing neurons to a holding potential close to their $\text{ex vivo}$ resting potential, unmasked by the near complete absence of spontaneous network activity (with the perfusate temperature and extracellular $[\text{K}^+]$, $[\text{Mg}^{2+}]$ and $[\text{Ca}^{2+}]$ used in these experiments, crucial determinants of cell and network excitability (Sanchez-Vives and McCormick, 2000; MacLean et al., 2005)). In the intact brain however, largely due to the greatly enhanced rates of network input, concurrent depolarisation and shunting of input resistance occurs (Borg-Graham et al., 1998). Neurons can often be depolarised by tens of millivolts compared to estimates of resting potential from slices - both during UP states observed during quiet wakefulness (Crochet and Petersen, 2006; Gonçalves et al., 2013), and more canonically during sleep and anaesthesia (Steriade and Nuñiez, 1993; Crunelli and Hughes, 2010).
A potential criticism of this experiment therefore is that genotype-specific change to input resistance and impedance spectra will become reduced under depolarised membrane conditions expected in vivo or during transient UP states as observed in slice preparations (e.g. Sanchez-Vives and McCormick 2000; Beierlein et al. 2002; Gibson et al. 2008). To address this possibility, both D.C. input resistance and impedance spectra were characterised in a sub-sample of neurons at holding potentials ±10mV of -60mV (Figure 3-10a). In this dataset, at each holding potential Fmr1-KO neurons showed a slight trend toward increased D.C. R_{membrane} compared to neurons from wildtype littermates (sub-significant as measured from -5pA D.C. current steps, Figure 3-10b).

**Figure 3-10** Elevated input resistance and altered impedance profiles characteristic of Fmr1-KO L4 excitatory neurons appear robust when neurons are de/hyperpolarised ±10mV from resting potential.

a). R_{input} and impedance profiles calculated at holding potentials of -50mV (top), -60mV (centre) and -70mV (bottom). R_{input} was estimated by applying 500ms duration -5pA hyperpolarising steps in current clamp from holding potential indicated. Impedance profiles were estimated by applying 20s rising frequency sweeps between 0.5Hz-100Hz. Amplitudes were calibrated to yield +10mV depolarisation with 0.5Hz oscillations. Data shown is mean±SEM. N: Fmr1^{+/Y}=10-12 neurons, Fmr1^{−/Y}=8-10 neurons. N.B. Fmr1^{−/Y} impedance profile at \(V_{\text{hold}}=-50\text{mV}\) is contaminated by AP-firing.

b). Mean±SEM (solid lines) D.C. input resistance for neurons in a). re-ploted to highlight effect of holding potential for individual neurons (dotted lines).

c). Mean±SEM steady state I/V relationship for Layer 4 excitatory neurons calculated by applying 500ms duration current steps from a holding potential of -70mV. Note reduction in rate of depolarisation at approximately -50mV. Dotted lines indicate linear fits to individual data points for current injection of ±40pA. Estimated slope resistance in this region was significantly elevated in Fmr1^{+/Y} neurons (p<0.0001, extra sum-of-squares F-test, F ratio=63.6, Fmr1^{+/Y}: N=13, \(R^2=0.96\); Fmr1^{−/Y}: N=9, \(R^2=0.98\).
D.C. $R_{\text{input}}$ / Impedance (MΩ)

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>$V_{\text{holding}}$</th>
<th>D.C. Input Resistance (MΩ)</th>
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</thead>
<tbody>
<tr>
<td>$-70$</td>
<td>$665\pm19$ MΩ</td>
<td></td>
</tr>
<tr>
<td>$-60$</td>
<td>$479\pm10$ MΩ</td>
<td></td>
</tr>
<tr>
<td>$-50$</td>
<td></td>
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</table>

Steady-State Potential (mV) vs. Injected Current (pA)
Significantly elevated impedance in frequency ranges between 0.5-10Hz was observed in Fmr1-KO neurons at each holding potential, however the frequency ranges over which the genotype-specific effect was observed varied according to holding potential. Widest frequency range differences were observed at -60mV as previously described. Holding neurons at -70mV reduced the frequency range over which impedance was significantly elevated in Fmr1-KOs over littermate neurons to 0.5-2Hz (c.f. 0.5-3Hz at -60mV).

Calculated impedance spectra were “flatter” for neurons of either genotype when held at -50mV – i.e. less pronounced effect of increasing frequency was observed on impedance. Despite loss of significant elevated mean impedance at lowest frequencies (i.e. down to 0.5Hz) in Fmr1-KO neurons, an impedance peak between 3-8Hz was observed in each neuron tested. This range was significantly different between genotypes since this behaviour was not observed in any wild-type neurons tested, and likely represents contamination from regular spike AHPs rather than a resonant band, since Fmr1-KO neurons were observed to fire more APs during ZAP injection when depolarised to this potential. Finally, in a separate group of excitatory neurons, D.C. input resistance was measured by depolarising neurons away from a –70mV holding potential with 10pA incremental current injections (Figure 3-10c). Membrane potential measurements were averaged over the last 10ms of a 500ms depolarisation (rejecting trials with spikes fired in the last 200ms). A strong linear trend confirmed that the steady-state (i.e. approximately D.C.) input resistance was consistently
elevated in *Fmr1*-KO neurons at potentials from -90mV to approximately -50mV. When further depolarised above -50mV, a shunting effect was observed in neurons of both genotypes, presumably due to the opening of voltage-gated channels. In *Fmr1*-KO neurons the departure away from the linear subthreshold input resistance was less pronounced until higher current injection amplitudes, suggesting that input resistance was maintained elevated over that of wild-type neurons even at potentials close to firing threshold\(^{22}\). As such, elevated input resistance could be expected to impact synaptic integration by *Fmr1*-KO excitatory Layer 4 neurons in the intact brain during the sustained membrane depolarisation observed *in vivo* during awake sensory processing (Crochet and Petersen, 2006).

### 3.5.6.3. Frequency following of spikes to sinusoidal inputs

To examine how the elevated input impedance of *Fmr1*-KO neurons to low frequency oscillatory inputs would introduce changes to their AP firing behaviour, responses to repeated cycles of injected sinusoidal current were recorded whilst cells were maintained depolarised close to threshold. Cells were held 5mV below firing threshold, empirically determined for each neuron by injecting bias current\(^{23}\). 5s of constant amplitude, constant frequency sinusoidal current was injected into the soma via patch pipette (Figure 3-11). Responses to frequencies between 0.5 and 50Hz were examined (Figure 3-11a).

\(^{22}\) N.B. firing threshold was not significantly different between genotypes at rheobase – see Figure 3-4.

\(^{23}\) Cells in which this holding value drifted by >2mV in either direction between trials were rejected.
**Figure 3-11** Entrainment of action potential firing by rhythmic oscillatory current waveforms in layer 4 excitatory neurons.

**a).** Single example recordings. Static frequency sinusoid current waveforms (50pA peak-peak amplitude) were injected into current-clamped layer 4 excitatory neurons. Neurons were maintained depolarised at -45mV (5mV below mean threshold – not significantly different between genotypes).

**b).** Mean±SEM number of APs evoked (i.e. “rotation number”) by each oscillatory cycle for waveform frequencies between 0.5 – 50Hz. Measurements are averaged over 5 second recording, repeated three times. *Fmr1*<sup>+/Y</sup> neurons fired significantly more APs per cycle than littermates at frequencies between 0.5 – 4Hz (p<0.05, t-test, n: *Fmr1*<sup>+/Y</sup> = 5, *Fmr1*<sup>+/Y</sup> = 8). Inset: Expanded view of frequency range over which *Fmr1*<sup>+/Y</sup> neurons lock to one AP per cycle. The range of frequencies across which 1 AP/cycle was elicited was narrower in *Fmr1-KOs* (4-7Hz in *Fmr1*<sup>+/Y</sup> neurons vs. 7Hz in *Fmr1*<sup>+/Y</sup> neurons, P>0.05, one-sample t-test)

**c).** Mean±SEM AP failure probability (i.e. fraction of oscillatory cycles where APs were not evoked) as a function of input waveform frequency. No significant difference was observed between genotype for any frequency between 0.5 – 50Hz (p>0.05, t-test, n: *Fmr1*<sup>+/Y</sup> = 5, *Fmr1*<sup>+/Y</sup> = 8).
**a**

<table>
<thead>
<tr>
<th>Sinusoid Frequency (Hz)</th>
<th>Fmr1(^{+/Y})</th>
<th>Fmr1(^{-/Y})</th>
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<tbody>
<tr>
<td>1 Hz</td>
<td></td>
<td></td>
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<tr>
<td>10 Hz</td>
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<td></td>
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<tr>
<td>25 Hz</td>
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**b**

![Graph showing no. APs/cycle vs. Sinusoid Frequency (Hz)]

**c**

![Graph showing AP Failure Probability vs. Sinusoid Frequency (Hz)]
For low frequencies sinusoids (typically below 4Hz), wild-type neurons typically fired more than on AP per cycle (i.e. “rotation no” >1), before locking into a stable response of one AP per cycle for a range frequencies between 4-7Hz. Higher frequencies could not be followed faithfully with 1 AP/cycle (“1:1 locking“), leading to frequency-progressive dropout such that mean numbers of APs/cycle dropped below one and progressed to <0.1 for frequencies above 20Hz (Figure 3-10b). Fmr1-KO neurons showed a substantially exaggerated spiking response to low frequency sinusoid injections, firing over three times the number of APs per cycle than evoked in wild-type neurons for lowest frequencies tested and significantly increased over wild-types within the 0.5-4Hz range.

Furthermore, the increased rate of firing in Fmr1-KO neurons in response to low frequency sinusoids dramatically contracted the range of frequencies over which 1:1 locking of AP firing to sinusoid cycle was achieved: whereas in wild-type neurons 1:1 locking was robust when input frequencies changed between 4-7Hz, it could only be stably achieved with 7Hz sinusoids in Fmr1-KO neurons (Figure 3-11b, inset). This effect was not contributed to by an increased probability of AP failure during higher frequency stimulation since mean per-cycle AP failure probability increased at a similar frequency-dependent rate in Fmr1-KOs and wild-types (Figure 3-11c).

Together these data demonstrate the effect of elevated membrane resistance in Fmr1-KO neurons upon their intrinsic responsiveness to somatic current injections in a frequency-dependent manner. In the intact brain, excessive firing that has been reported during (“delta”, ~0.8-4 Hz)
slow-wave sleep in *Fmr1*-KO mice of similar ages (Gonçalves et al., 2013) could be contributed to by this phenotype. Additionally, it is likely that the reduced tolerance of varying input frequency on the faithful locking of output firing in the present data (“low theta”, 4-7Hz) will disrupt computations that dependent upon neocortical theta modulation (Sirota et al. 2008), considered essential for memory consolidation during sleep (e.g. in the barrel cortex: Vijayan et al. 2010).

3.5.7. Altered spontaneous synaptic input to Layer 4 excitatory *Fmr1*-KO neurons at P10-11

In order to understand how altered cell-intrinsic excitability contributes to changes in network processing, it is essential to consider the degree of connectivity between neurons. One broad measure of “synaptic connectivity” can be obtained by examining global spontaneous synaptic transmission onto a postsynaptic neuron. In the largely quiescent *ex vivo* slice preparation, spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs respectively) arise predominantly from spontaneous quantal release, rather than driven by neuronal firing, with a rate dependent on calcium concentration and temperature (Katz and Miledi, 1969; Schneggenburger and Neher, 2000). Under these conditions, the rate of detected spontaneous postsynaptic currents reflects both the probability of presynaptic release and the number of synaptic contacts, whilst the current amplitude reflects the number of postsynaptic neurotransmitter receptors at the synaptic cleft (as a proxy for the synaptic strength) and ultimately is determined by the quantal neurotransmitter content of the released vesicle. Previous studies of neurodevelopmental
disorders have presented changes to spontaneous synaptic transmission in slice recordings from mutant mice (e.g. Down Syndrome: Chakrabarti et al. 2010 and Fragile X Syndrome: Vislay et al. 2013) as general markers of altered synaptic physiology. Potentially representing an additional role however, intracellular signalling cascades and gene expression are sensitive to the rate of spontaneous synaptic input independent of that via spike-driven transmission. For example, the effect of blocking the responses to spontaneous synaptic transmission with NMDAR antagonists on dendritic CaMKII and MAPK activation is stronger than that of blocking spike-based transmission with tetrodotoxin (Murphy et al., 1994). Such a mechanism has been suggested as an essential homeostatic link to providing epigenetic modulation of gene expression (Day and Sweatt, 2011), likely providing modulation by cascades downstream of the NMDA receptor (Espinosa and Kavalali, 2009). Furthermore it is essential to appreciate a physiological role for the finely balanced rate of inhibitory to excitatory spontaneous transmission: Asynchronous inhibitory release can provide a substantial contribution to inhibitory tone in postsynaptic excitatory neurons (Lu and Trussell, 2000), whilst at specialised synapses, spontaneously released uniquantum transmission can reliably drive AP firing in electrotonically-compact cerebellar stellate neurons (Carter and Regehr, 2002).

In order to examine spontaneous synaptic transmission onto Fmr1-KO excitatory neurons in Layer 4, whole-cell recordings were made with an internal solution containing CsMeSO₄ and the sodium channel blocker QX-314. With this approach, by blocking AP-related K⁺ and Na⁺ voltage-gated conductance respectively, neurons could be voltage-clamped equally well
at the reversal potential for GABAergic (-70mV) and glutamatergic (0mV) ionotropic transmission, allowing both excitatory and inhibitory spontaneous inputs to be examined sequentially from the same cells. **Figure 3-12** shows example voltage-clamp recordings of spontaneous synaptic events recorded from Layer 4 excitatory neurons.

**Figure 3-12** Spontaneous excitatory (sEPSC) and inhibitory (sIPSC) currents recorded in L4 excitatory neurons isolated by voltage-clamping at -70mV and 0mV respectively using a CsMeSO₄–based internal solution (including QX-314).

(a). Example recordings (left) and example cell-average detected responses (right). Average traces are aligned by detected current peak. Grey: individual tagged events, colour: mean±SEM spontaneous currents. (b). Left: Schematic recording configuration. Right: A sub-significant trend towards reduced current frequency was observed for both sEPSCs and sIPSCs in Fmr1⁺/⁻ neurons. Data points indicate individual neurons.
sEPSCs and sIPSCs were recorded for 60s and events were detected automatically using a template-based search method. Recordings from both wild-type and Fmr1-KO neurons displayed strong inter-cell variability in the number of detected sEPSC and sIPSC per 60s, however a slight trend towards a reduced mean rate in Fmr1-KOs was observed for both (Fmr1$^{+/Y}$ vs. Fmr1-KO (n=21 and 17 cells, respectively, max of 3 per animal); sEPSCs: p=0.72; sIPSCs: p=0.74, Mann-Whitney). It is unlikely that false negative rate is different between genotypes, since the space-clamp provided by the Cs$^+$-based internal solution was excellent, and root-mean-square (r.m.s.) current noise levels were rigorously maintained <8pA for all recordings. Furthermore, dendritic damage following slice preparation is unlikely to account for this degree of variability. Such a wide inter-cell variability therefore could reflect the strongly diverse, heavy-tailed distribution of synaptic connectivity rates previously reported between excitatory neurons in layer 4 (Feldmeyer et al., 1999; Petersen and Sakmann, 2000; Lefort et al., 2009; O’Connor et al., 2010; Ashby and Isaac, 2011). Compared to frequencies, sEPSC and sIPSC amplitudes were less diverse between cells, as characterised by the modal size for each recorded cell. In nearly all recorded wild-type excitatory neurons, the modal sIPSC amplitude (and intra-cell distribution) was larger than that of sEPSCs, with a population mean sIPSC:sEPSC amplitude ratio significantly elevated above unity (Figure 3-13). In Fmr1-KO neurons however, a slight reduction in the modal sIPSC amplitude abolished this trend such that the average inhibitory/excitatory tone balance was not significantly different from unity. Repeating this analysis for the rate of spontaneous transmission
showed no effect of genotype, where conversely both genotypes showed higher numbers of sEPSC than sIPSCs recorded during 60s (Figure 3-13ai). Finally, the decay kinetics of EPSCs and sIPSCs were compared between genotypes (Figure 3-13b). This parameter can be informative about the neurotransmitter receptor subtypes involved, and is of relevance here since changes in GABA<sub>a</sub> receptor expression patterns and decay kinetics have previously been reported in the Fmr1-KO mouse (D’Hulst et al., 2006; D’Hulst and Kooy, 2007; Paluszkieiwicz et al., 2011; Vislay et al., 2013). Single exponential functions were fit to sEPSC and sIPSC decay. To compare kinetics between genotypes, the intra-cell distributions and modal decay constant were considered. Population-binned distributions were significantly different between genotypes for both sEPSCs and sIPSCs. Modal sIPSC decay constants were significantly faster in Fmr1-KO neurons, while despite a slight increasing trend no significant effect was observed for sEPSC kinetics. Interestingly, the faster sIPSC kinetics are in juxtaposition to a recent report at the same age from the Huntsman laboratory, where sIPSC recorded from excitatory neurons in basolateral Amygdala in Fmr1-KO slices decayed significantly slower than those in wild-type neurons (Vislay et al., 2013).
Figure 3-13 Amplitude, frequency and kinetics of sEPSC and sIPSCs recorded in L4 excitatory neurons presented in Figure 3-12.

a). Intra-cell average statistics comparing balance of average (modal) amplitude (i) and frequency (iii) of sIPSCs vs. sEPSCs. Inset histograms (iii, above) show cumulative amplitude histograms of individual neurons (thin lines) and mean±SEM histograms (shaded regions). Distributions were not significantly different between genotypes for either sEPSCs or sIPSCs.

(i) Data points indicate individual neurons. Solid lines are linear fits to the data. Dotted line marks unity. For Fmr1+/Y neurons, the trend for modal sIPSC:sEPSC amplitude ratios was significantly different from unity, but not in Fmr1-/- neurons (Extra-sum-of-squares F test, $F_{17}$: $p=0.03$, best fit to slope vs. slope=1: $p=0.003$, best fit to Y-intercept vs. Y-intercept=0 (n=21). Fmr1-/-: $p=0.3$, best fit to slope vs. slope=1: $p=0.2$, best fit to Y-intercept vs. Y-intercept=0 (n=17). The same comparison was insignificant for both genotypes for number of events detected per 60s (ii).

b). Decay kinetics of sIPSCs and sEPSCs. Left: cumulative distributions of individual current decay time constants fit by single exponential decay functions (example shown in inset). Heavy lines indicate population-binned histograms. $\tau_{\text{decay}}$ distributions were significantly different between genotypes for both sEPSCs and sIPSCs ($p<0.05$, K-S test, n: sEPSCs and sIPSCs of 17 neurons binned for each genotype). Right: Data reanalysed by fitting decay kinetics to average currents for each neuron (e.g. Figure 3-12a, right). Cell average sIPSCs decayed significantly faster in Fmr1+/Y neurons compared to those from littermates ($p<0.05$, t-test, n: 17 neurons for each genotype). Decay kinetics of sEPSCs recorded from the same neurons were not significantly different between genotypes.
(iii) Cumulative fraction

![Graphs showing cumulative fraction of modal EPSC and IPSC amplitudes.](image)

(a) i. Modal IPSC amplitude (pA) vs. Modal EPSC amplitude (pA)

![Graph showing correlation between modal IPSC and EPSC amplitudes.](image)

ii. No. IPSCs /60s vs. No. EPSCs /60s

![Graph showing correlation between number of IPSCs and EPSCs.](image)

(b) Cumulative fraction vs. Spontaneous current decay constant (ms)

![Graph showing cumulative fraction and spontaneous current decay constant.](image)

Cell-average event decay constant (ms)

![Graph showing cell-average event decay constant.](image)
Characterisation of sEPSCs onto layer 4 excitatory neurons was repeated with a larger dataset using a KMeSO₄-based internal solution (Figure 3-14). These recordings were obtained as a by-product of experiments that characterised firing properties and circuit activity in Fmr1-KO layer 4.

Figure 3-14 A larger dataset of L4 excitatory neurons recorded using K⁺-based internal solution shows a significant reduction in spontaneous EPSC frequency and altered amplitude distribution in Fmr1⁻/⁻ neurons.

a). Left: One second of example gap-free voltage-clamp recordings (-70mV holding potential) for each genotype. Right: 60-second average sEPSCs for recordings shown on left, aligned by current peak.

b). Left: significantly fewer sEPSCs per 60 seconds were detected in recordings from Fmr1⁺/⁺ neurons. Note large range of detected input frequencies between neurons of both genotypes. Right: no statistically significant change between genotypes was observed for sEPSC decay kinetics for 60s average sEPSC. (t-test, N: Fmr1⁺/⁺ = 61 neurons, Fmr1⁻/⁻ = 81 neurons).

c). Cumulative distributions of amplitudes (left) and inter-event intervals (IEIs, right) of sEPSC onto L4 excitatory neurons recorded during a 60s window. Thin line are cumulative distributions of individual neurons, Thick lines show the bin-by-bin mean±SEM of bootstrap-resampled distributions in which each neuron contributes 20% of events recorded in 60s to population dataset. Mean amplitude and IEI distributions were significantly different between genotypes (K-S test, N: Fmr1⁺/⁺ = 61 neurons, Fmr1⁻/⁻ = 81 neurons). Statistically significant differences are similarly obtained by the same tests if alternatively all events from each cell are pooled into a population distribution.

24 But always obtained before any other single-cell or network stimulation was performed.
**a**

- $Fmr1^{+/+}$
- $Fmr1^{-/-}$

**b**

- No. sEPSCs detected 60s
- Cell mean sEPSC decay tau (ms)

**c**

- Cumulative fraction (%)
  - sEPSC amplitude (pA)
  - sEPSC Inter-event interval (ms)

*p* < 0.001
Comparable to data from recordings with Cs-based internal presented previously, space clamp was good due to the electrical compactness of this cell type; neurons could be voltage clamped at 0mV and prevented from firing. However, to avoid adverse effects on physiology in these experiments, only data from sEPSCs was obtained (i.e. holding neurons at -70mV). Under these conditions, R.M.S. current noise compared favourably to that of the previous dataset, and it is unlikely that a significant increase in false negative rate (missed detection of small events) was introduced (compare example traces and smallest detected K⁺ sEPSCs (Figure 3-14b) to Cs⁺ sEPSCs (Figure 3-12); no hard amplitude threshold criterion was imposed by template-matching algorithm). Furthermore, space-clamp errors are theoretically lower in Fmr1-KO neurons since the input resistance is higher on average in cells from this genotype.

In this larger dataset, the trend towards lower rate of detected sEPSCs/minute in Fmr1-KO neurons reported above attained statistical significance (Figure 3-14a,b). The population average sEPSC amplitude distributions were also significantly different between genotypes, despite showing no change in mean or median values for either population-binned or individual-neuron statistics. Population mean±SEM cumulative amplitude histograms for Fmr1-KO neurons were steeper and right-shifted in the lowest quartile compared to wild-type neurons, suggesting a reduced contribution from small amplitude synaptic events. This effect could be interpreted as a reduced range of synaptic strengths onto the “average” Fmr1-KO neuron. Finally, as in the previous dataset a trend
towards slower average sEPSC decay kinetics was observed in \textit{Fmr1-KO} neurons.

Together, these data demonstrate that \textit{Fmr1-KO} excitatory neurons in the present study receive spontaneous synaptic current inputs at a reduced rate compared to wild-type neurons, and that the average balance of spontaneous GABAergic to glutamatergic current strengths received by individual neurons is reduced. This ratiometric change in sIPSC/sEPSC currents appears specific to their amplitude since the trend towards reduced rate in the Cs\(^+\)-based recording dataset (\textbf{Figure 3-14}) appeared proportionate for sIPSCs and sEPSC in \textit{Fmr1-KOs}. The shift towards faster sIPSC decay kinetics is interesting since a developmental transition towards faster GABAergic synaptic kinetics has been previously reported and linked to onset of alpha-1 GABA\(_\alpha\) receptor subunit expression (Laurie and Wisden, 1992; Hollrigel and Soltesz, 1997; Eyre et al., 2012). This would argue against a developmental delay in stoichiometric maturation of GABAergic synaptic conductances in the present data, contradicting work showing the opposite in recent literature in other brain areas at the same age (D’Hulst et al., 2006; Vislay et al., 2013). It is important to note however that altered decay kinetics could alternatively result from altered synaptic locations – a faster mean sIPSC decay constant could be indicative of a majority of GABAergic synapses arising close to the soma, thus experiencing comparatively less space-clamp distortion (Spruston et al., 1994). Such a scenario might arise from fewer inputs from distal dendrite-targeting Som\(^+ve\) interneurons (providing “feedback” inhibition), in conjunction with either unchanged or more elaborate basket inhibition
from PV⁺⁺ interneurons (providing feed-forward inhibition) (Beierlein et al., 2009). If so, the balance in inhibitory tone afforded by feed-forward and feed-back inhibition would be expected to shift, such that computation would be differentially warped depending on whether synaptic inputs were brief and edge-like, or sustained – dictated by the differential short-term plasticity at these two sources of inhibition.

When determining the cause of the reduced rate of spontaneous release, it is impossible to distinguish between changes in presynaptic release probability and changes in connection probability through examining this data alone. However, since layer 4 excitatory neurons are preferentially innervated predominantly by thalamocortical terminals and within-barrel excitatory neighbouring neurons (White and Rock, 1979, 1981; Petersen and Sakmann, 2000; Lefort et al., 2009; Ashby and Isaac, 2011), changes in excitatory connectivity can, from an anatomical prior, be largely confined to two putative presynaptic origins. It is unlikely that changes in thalamocortical inputs solely account for these reductions since they are outnumbered approximately five-fold by intracortical inputs (White and Rock, 1981; Bruno and Sakmann, 2009). Furthermore, since excessive short-term depression was observed at thalamocortical inputs onto Fmr1-KO neurons (see chapter 4), it is more likely that release probability at this synapse is increased rather than decreased.

A previous study found developmentally delayed silent synapses at thalamocortical inputs to Layer 4 neurons in Fmr1-KOs (Harlow et al.,

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25 N.B this represents a first approximation since these references are to studies performed on older animals with mature connectivity.
2010), consistent with the effect of whisker deprivation during the critical period for thalamocortical plasticity (Isaac et al., 1997). The observation that developmental sensory deprivation similarly causes a delayed emergence of excitatory-excitatory connectivity in layer 4, similarly involving delayed “AMPAfication” of silent intracortical synapses suggests a similar defect could occur at Fmr1-KO intracortical synapses. While Fmr1-KO excitatory-excitatory paired connections (or synaptic silence, intracortical or otherwise) were not tested in the current study due to their relative scarcity in wild-type neurons at this age (p(connection) <30%, Ashby and Isaac 2011), it is plausible that a reduction in these connections plays a significant role in the reducing the intracortical synaptic input to layer 4 neurons, and by extension, the functional recurrent architecture of the barrel excitatory circuit (Ashby and Isaac, 2011).

Paired connections were however tested between Fast-Spiking interneurons and excitatory neurons in Fmr1-KO layer 4 and found to be dramatically reduced in connection probability, but possessing excessive short-term synaptic depression (discussed later – see Figure 3-20, 21) Since FS neurons provide a significant fraction of inhibitory synaptic input to Layer 4 excitatory neurons, such a bidirectional divergence could explain why no overall significant reduction in sIPSC frequency was observed in Fmr1-KO neurons.
3.5.8. Interaction between cell-intrinsic excitability and spontaneous synaptic input to excitatory neurons in Layer 4

Previous studies examining network properties of the Layer 4 recurrent excitatory circuit have noted that the statistics of synaptic connectivity and sensory-evoked network responses conform to strongly heavy-tailed, non-Gaussian distributions (Feldmeyer et al., 1999; Petersen and Sakmann, 2000; Lefort et al., 2009; O’Connor et al., 2010; Ashby and Isaac, 2011). Others who have studied emergent intra-network heterogeneity suggest that clustered, “small-world” network topology is computationally advantageous (Bullmore and Sporns, 2009; Padmanabhan and Urban, 2010; Angelo et al., 2012; Litwin-Kumar and Doiron, 2012; Tripathy et al., 2013). Furthermore, correlations between intrinsic neuronal properties and physiological function have been demonstrated in diverse brain regions (e.g. theta oscillation frequency varying with cell position within entorhinal cortex as a function of an HCN channel expression gradient: Garden et al. 2008a; Giocomo and Hasselmo 2009, homotypy of HCN expression levels between co-processing olfactory bulb mitral neurons: Angelo et al. 2012). Similar observations have been made at the level of specialisations in synaptic physiology (e.g. lower variance of connection strengths in densely-connected recurrent clusters, driving mean weight towards convergence: Perin et al. 2011). A disrupted relationship between the statistics of synaptic input and intrinsic postsynaptic neuronal properties would thus be expected to contribute to network dysfunction.
This is next addressed by comparing intrinsic and synaptic physiology between genotypes on a within cell basis.

3.5.8.1. Correlations between intrinsic excitability and spontaneous synaptic input are altered in Fmr1-KO excitatory neurons

In the present study, a significant negative correlation was observed between the rate of spontaneous EPSCs recorded from wildtype neurons and their input resistance (Figure 3-15ai). This relationship was significantly weaker in Fmr1-KO neurons compared to those from wild-type mice, such that more excitable Fmr1-KO neurons were observed to have unusually high rates of spontaneous synaptic input. No significant correlation was observed between input resistance and sEPSC amplitude for either genotype (Figure 3-15aii). Interestingly, whilst in wild-type neurons no correlation was observed between the sEPSC rate and amplitude on an individual neuron basis, a slight but significant positive correlation was present in Fmr1-KO neurons (Figure 3-15aiii).

An immediate dismissal of this effect as artefactual could be that better space clamp due to elevated input resistance in Fmr1-KOs would improve resolution of smaller events, however, as previously discussed this scenario is incompatible with the findings presented above, particularly since sEPSC rate was found to be decreased in Fmr1-KO neurons without a corresponding shift in detection threshold or change in voltage clamp noise. Indeed, a positive sEPSC frequency vs. input resistance trend might be expected if this were the case, since elevated space clamp would reduce attenuation of small/distal inputs via voltage clamp error. This effect has
been explored previously: For a simplified ball-and-stick model neuron, a ten-fold increase in dendritic input resistance has minimal additional effect on attenuating distal (i.e. several electrotonic lengths away) synaptic conductance as recorded at the soma. Similar results are obtained when examining passive dendritic attenuation of synaptic inputs in morphologically-realistic model neurons (Spruston et al., 1993, 1994). Furthermore, laser uncaging of glutamate onto dendrites produces somatic currents with amplitudes that are independent of photolysis position, even in apical dendrites of morphologically-complex CA1 (Pettit and Augustine 2000; glutamate and GABA uncaging) and cortical L5 neurons (Frick et al., 2001).

**Figure 3-15** Interaction between intrinsic excitability and spontaneous excitatory synaptic input in layer 4 excitatory neurons:

a). Correlations between rate/amplitude of sEPSCs and neuronal input resistance. Points are individual neurons, fits are linear regression trends, and black symbols are mean±SEM markers. Pearson correlation between plotted parameters and slope of trends are shown below each figure. Difference in slope between genotypes was tested regardless of whether or not a significant inter-parameter correlation was observed in both. Asterisks indicate significantly different correlation slopes between genotypes (Extra-sum-of-squares F test, N (neurons): Fmr1+/Y =61, Fmr1−/Y =81).

b). Elevated instability in spontaneous membrane potential fluctuations at resting potential recorded in Fmr1−/Y neurons. Left: Ten seconds of example spontaneous membrane potential recorded at resting potential in the absence of electrical/synaptic stimulation (in voltage-follow “I=0” mode). Centre: Mean-shifted Vmembrane histograms calculated from 60s of recording from layer 4 excitatory neurons. Light traces show data, heavy traces are Gaussian fits. Black lines mark 1x standard deviation (68.27% of maximum) for each fit. Right: Voltage instability (i.e. population distribution of Vmembrane standard deviations as shown left) was significantly increased in Fmr1−/Y neurons. (P<0.05, t-test, N: Fmr1+/Y =10 neurons, Fmr1−/Y =8 neurons).

c). Multi-taper power spectral density of membrane potential oscillations for recordings shown above (time-bandwidth product = 3, 5 tapers). Data are mean±SEM. Fmr1+/Y membrane potential fluctuations showed significantly elevated power at frequencies between 0.2-30Hz (p<0.05, t-tests performed on mean power between log-spaced bins. N (neurons): Fmr1+/Y =10, Fmr1−/Y =8).  

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**a**  
**sEPSCs:**  

![Plot](image1)

- Input Resistance (MΩ) vs. No. /60s.
- Modal amplitude (pA) vs. Input Resistance (MΩ)
- No. /60s.

- $r = -0.33$ (p<0.001), slope = -0.4
- $r = -0.27$ (p<0.01), slope = -0.1
- $r = -0.008$ (n.s.), slope = -0.0002
- $r = -0.006$ (n.s.), slope = -0.007
- $r = -0.09$ (n.s.), slope = -0.002
- $r = -0.31$ (p<0.05), slope = 0.007

**b**  
**sEPSPs:**  

![Waveform](image2)

- $Fmr1^+/Y$
- $Fmr1^{-}/Y$
- Membrane potential frequency spectra:

**c**  
**Membrane potential frequency spectra:**  

![Spectrum](image3)

- $P<0.05$
With this in mind, it is important to note that the principle synaptic inputs to layer 4 excitatory neurons - thalamocortical and recurrent layer 4 connections – form synapses onto spines that are located predominantly within 100 μm of the soma (Ashby and Isaac 2011; White and Rock 1979, 1981; Connors B., personal communication; Rah JC. and Isaac JTR., personal communication; Konnerth A., personal communications). The electrotonic structure (i.e. dendritic branching and length) and dendritic spine density/distribution are grossly normal in young Fmr1-KO layer 4 excitatory neurons (Harlow et al., 2010; Henderson et al., 2012; Till et al., 2012) thus it is likely that shifts in synaptic location contribute only minimal effect to spontaneous input. Finally, no increase in sEPSC decay time constant was observed in the Fmr1-KO dataset, suggesting that whole-scale changes to dendritic filtering of sEPSC kinetics were not occurring.

Together, this suggests that the relaxed negative relationship between input resistance and spontaneous input frequency, and positive correlation between sEPSC rate and modal amplitude in Fmr1-KO neurons represent a biological effect and could impact the stability of the membrane potential during spontaneous synaptic input. In addition to influencing input-output gain by modulating voltage noise as discussed previously, this effect also raises intriguing questions about the state of homeostatic plasticity in young Fmr1-KO neurons.
3.5.8.2. Membrane potential instability is greater at resting potential in *Fmr1-KO* excitatory neurons

To directly examine the impact of elevated neuronal excitability in *Fmr1-KO* neurons on responses to spontaneous synaptic transmission without biasing membrane potential with current injections, membrane potential fluctuations were recorded from neurons at resting potential in voltage-follow configuration (Figure 3-15b). Membrane potential stability at resting potential was significantly reduced in *Fmr1-KO* neurons, as indicated by greater $V_m$ standard deviation during sixty seconds of spontaneous input. Furthermore, frequency decomposition of these recordings showed significantly elevated spectral power for low frequency bands between 0.2Hz and 30Hz (Figure 3-15c). This finding agrees with the above results from impedance profiling experiments using injected sinusoidal current, and is consistent with the increased attenuation of fast synaptic events, but heightened sensitivity to slow fluctuations predicted as a result of elevated dendritic input resistance (Spruston et al., 1993, 1994; Magee, 2000).

3.5.8.3. Modelling the interaction between neuronal excitability and spontaneous input in *Fmr1-KOs*

One potential explanation of this effect could be that excitatory synaptic transmission (rate and/or amplitude) is linked to postsynaptic neuronal excitability via homeostatic regulation, and that loss of FMRP disrupts this mechanism. In this scenario, synaptic activity has been shown to activate intracellular signalling cascades (e.g. NMDAR activating CaMKII activating MAPK, leading to epigenetic modulation of gene expression and
ion channel synthesis: Murphy et al. 1994; Day and Sweatt 2011), providing the cell with a feed-forward control loop to regulate its excitability. An antagonistic situation might involve control of plastic changes to synaptic strength through the introduction of voltage dependence to spine $[\text{Ca}^{2+}]$ dynamics (O’Leary et al., 2009; O’Donnell et al., 2011), or potentially through trans-synaptic or retrograde signalling. Thus, the inverse relationship of sEPSC rate to input resistance in wild-type neurons might arise through such mechanisms, and a weakened correlation as observed in Fmr1-KO neurons could reflect homeostatic disruption following loss of FMRP. Indeed, the elevated basal protein translation levels (reviewed in Bear et al. 2007) and plethora of intracellular signalling cascades disrupted in the Fmr1-KO brain (Brown et al., 2001; Darnell et al., 2001, 2011) are highly likely to impact homeostatic control mechanisms$^{26}$.

To explore the physiological consequences of disruption to a hypothetical homeostatic link between neuronal input resistance and rate of spontaneous synaptic input in the present data, a computational modelling approach was adopted. Here, a morphologically realistic NEURON model was built from a biocytin-filled layer 4 excitatory neuron fixed after recording, then reconstructed using confocal microscopy and semi-automated neurite tracing (Figure 3-16a). 200 identical excitatory synaptic inputs$^{27}$ were randomly distributed across the dendritic arbour and

$^{26}$ Similar discussions exist in the literature: (Tessier and Broadie, 2008; Soden and Chen, 2010; Henry, 2011; Brager et al., 2012; Vislay et al., 2013).

$^{27}$ Adult Layer 4 Ex. neurons have ~2000 dendritic spines, however these 200 sites were chosen to represent the fraction considered to arise from TC inputs (Bruno and Sakmann; 2009).
stimulated with independent Poisson-rate inputs matching the genotype mean frequency of wild-type sEPSCs (black markers in Figure 3-15ai).

Using this (stylised and conceptual) model, the interaction between input resistance and rate of sEPSCs was investigated by independently varying a global (i.e. dendritic and somatic) passive leak conductance and synaptic stimulation rate proportionate to genotype means observed either in wild-type or Fmr1-KO neurons. This approach yielded four hypothetical sEPSC rate vs. input resistance combinations. Figure 3-16b shows ten seconds of simulated somatic membrane potential responses to spontaneous synaptic inputs for each configuration. For comparison, dotted blue lines in each panel denote the mean potential for the “full wild-type“ condition (i.e. sEPSC rate and input resistance both matching genotype mean values for wild-type neurons, shown in blue). Figure 3-16c shows membrane potential histograms (as well as mean-shifted, removing depolarising offsets on the right).

For the wild-type condition (blue), approximately 10mV modal depolarisation from leak reversal potential (as a proxy for resting potential) and relatively narrow voltage histograms were observed during spontaneous synaptic input. However, when input resistance was increased to match Fmr1-KO genotype mean (red) while sEPSC rate was maintained at wild-type levels, approximately 20mV modal depolarisation was observed, coupled with a striking ~50% increase in $V_m$ histogram standard deviation. This result supports the hypothesis that elevated input resistance severely distorts the somatic voltage response to spontaneous current inputs that occur at “normal” wild-type rates.
The interaction between intrinsic membrane excitability and dendritically distributed synaptic input can affect the membrane potential stability of a morphologically realistic model excitatory neuron.

(a). Example workflow for generating an in silico model from a reconstructed whole-cell recording. An anatomically reconstructed neuron was stimulated by placing 200 identical model synaptic inputs randomly across the dendritic arbour. Dendrites and soma were given uniform leak conductance and membrane capacitance. Poisson rate spontaneous synaptic release caused fluctuation in the simulated membrane potential.

(b). Vmembrane for four simulation conditions, where passive leak conductance and number of detected sEPSCs/60s matched either Fmr1+/Y or Fmr1-/Y genotype means values, and conditions for each genotype where synaptic rate was proportionally reduced as detected in Fmr1-/Y recordings. Dotted line indicates mean potential for Fmr1+/Y simulation condition.

(c). Vmembrane distribution histograms for 10 seconds of simulated activity for conditions described above. Heavy lines are Gaussian fits. Voltage offsets are shown removed on the right. Note differential effects on voltage offset and Vmembrane fluctuation stability between simulation conditions.

(d). Multi-taper power spectral density calculated for simulated traces shown in b). (time-bandwidth product = 3, 5 tapers). Note that changes to either leak conductance or synaptic input frequency affect the frequency dependent gain simulated voltage responses.
a) L4 ex. neuron, biocytin fill (confocal z-projection)  Morphology reconstruction (Neuron Studio)  Passive biophysical model (Neuroconstruct/NEURON)

b) 10s of simulated Vm, driven by spontaneous synaptic input:

<table>
<thead>
<tr>
<th>$R_{input}$</th>
<th>+/Y</th>
<th>-/Y</th>
<th>-/Y</th>
<th>+/Y</th>
</tr>
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<tbody>
<tr>
<td>Input rate</td>
<td>+/Y</td>
<td>+/Y</td>
<td>-/Y</td>
<td>-/Y</td>
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c) Mean-Shifted

d) Vm power (dBm²/Hz)
Next, in addition to elevated input resistance, spontaneous stimulation frequency was reduced at all synapses to mimic a proportional reduction in the rate of sEPSCs as observed in *Fmr1-KO* excitatory neurons (green). In this scenario, both offset and standard deviation of voltage histograms were returned toward wild-type levels, however, over-correction was observed such that modal depolarisation was lower, and histograms were narrower than for the wild-type scenario. This undershoot likely arises from the error in experimental estimation of population mean values (note that a larger range of sEPSC rates for KOs in Figure 3-15ai). Finally, the effect of reducing sEPSC rate to mean *Fmr1-KO* levels was examined in combination with wild-type input resistance (black). Here, modal depolarisation from leak conductance reversal was less than the full wild-type simulation, but greater than that observed for the previous case of elevated input resistance with reduced sEPSC rate. A similar intermediate situation was observed for simulated V_m stability, where the voltage histogram was closest to that of the full wild-type simulation.

Interestingly, differences in the frequency power spectra of membrane potential were also observed between the four simulation conditions (Figure 3-16d). Reducing sEPSC rate had little effect on low-pass filter characteristics when input resistance matched mean wild-type values. However, elevating input resistance to *Fmr1-KO* mean while maintaining wild-type input rate introduced a strong increase in low frequency power density (below 10Hz), whereas simultaneously raising input resistance whilst reducing input rate led to broadband reduced spectral power, particularly at frequencies below 20Hz. This likely arises through
alterations in input integration introduced by a slower membrane time constant.

These simulations confirm that both neuronal input resistance and spontaneous input rate control membrane potential statistics independently and antagonistically. Interestingly, in addition to generating stronger depolarisation under the condition of wild-type input rate, the elevated input resistance as observed in Fmr1-KO excitatory neurons increases the membrane potential noise, which has previously been shown to modulate input-output gain during sparse coding (Chance et al., 2002; Azouz, 2005; Silver, 2010) This effect relies on the amplitude scaling of EPSP amplitudes and voltage noise, as well temporal scaling of EPSPs as an effect of longer time constant, together enhancing the integration of temporally dispersed inputs.

The extent to which these two controlling variables (input resistance and sEPSC rate) exert antagonistic control of the membrane potential will depend on their fine balance in individual neurons. Here, genotype means were assumed for simplicity. A more realistic scenario would explore covarying parameters to better capture the experimentally determined relationship between input resistance and sEPSC rate. Further simple extensions to this simulation could be to add active conductances and investigate the spike probability in response to co-incident synaptic input under varying background synaptic background rates and conductances, or to examine the role of dendritic input location on membrane potential deflection at the soma. It will also be interesting to pursue the correlation
between sEPSC amplitude and frequency detected specifically in \textit{Fmr1-KO} neurons – this is a subject of ongoing study.

Can these results offer insight into a potential homeostatic link between intrinsic neuronal excitability and frequency of spontaneous synaptic input, and potential changes in \textit{Fmr1-KOs}?

The choice of mean parameter values for these simulations makes drawing firm conclusions regarding a homeostatic link difficult, particularly given the high variability around these mean values. Nonetheless it is intriguing that the simulation that closest resembles the experimentally recorded membrane potential behaviour of \textit{Fmr1-KO} neurons is the second case (red in Figure 3-16), where elevated input resistance is uncompensated by a simultaneous reduction in input frequency. This would suggest that, at least in the cells sampled for the analysis presented in Figure 3-15, the effect of elevated input resistance is under-compensated by a reduction in synaptic input frequency. This combination is expected to promote excessive responsiveness to synaptic input and distort spatiotemporal integration, through the mechanisms discussed above.

3.5.9. Electrophysiological characterisation of Layer 4 fast-spiking inhibitory interneurons in \textit{Fmr1-KOs}

Neocortical fast-spiking GABAergic (FS) interneurons are predominantly Parvalbumin-positive (Cauli and Audinat, 1997) and provide strong basket (i.e. perisomatic) inhibition to, and are densely reciprocally targeted by principle excitatory neurons. Previous studies in \textit{Fmr1-KO} mice at older ages (e.g. Gibson et al. 2008) demonstrate a profound and persistent
unidirectional reduction in local excitatory drive onto FS interneurons in layer 4 of the barrel cortex. This and subsequent findings from the Gibson laboratory have provided crucial circuit level evidence that reduced local excitatory synaptic input to GABAergic interneurons contributes to inhibitory/excitatory imbalance in network activity in layer 4 (Gibson et al., 2008; Hays et al., 2011; Patel et al., 2013). No information on the physiology of Fmr1-KO FS interneurons at ages before two weeks old exists, however this developmental time-window is a period of intense physiological maturation of FS interneuron circuit and intrinsic properties (see discussion in 3.2.3). Furthermore, with the exception of resting potential, no changes to intrinsic properties of Fmr1-KO neurons have been reported in these studies.

To address this, patch recordings were made from putative Layer 4 FS neurons in brain slices prepared from Fmr1-KO and wild-type mice at P10-11. Figure 3-17 shows representative AP-firing behaviour of FS interneurons to rheobase and twice-rheobase current injections. Figure conventions follow those previously used for excitatory neurons in Figure 3-1. Passive electrophysiological characterisation, and analysis of spike shape at threshold was performed as before and is shown in Figure 3-18. In the present study, the results for intrinsic properties of Fmr1-KO Layer 4 FS interneurons follow a similar trend to those observed for excitatory neurons.

\[28\] See methods chapter (Ch. 2), for discussion of sampling strategy and Appendix 2 for validation of human classification system by unsupervised machine learning approach.
Figure 3-17 Altered AP firing properties of Layer 4 Fast-Spiking GABAergic interneurons (FS-INs) in Fmr1+/Y and Fmr1-/-Y neurons.

a). Example behaviour of neurons shown recorded in whole-cell current clamp configuration when depolarised from resting potential with 500ms square current step (shown below each recording). Coloured traces show responses to threshold (rheobase) current, black traces show responses to twice-rheobase current.

b). Phase plots for example recordings in a). Note slower speed of both AP depolarising and repolarising phases.
**Figure 3-18** Passive membrane properties and spike shape at threshold of Fast-Spiking interneurons in $Fmr1^{+/-}$ (blue) and $Fmr1^{-/-}$ (red) neurons.

Data are individual neurons, bars shows mean±SEM. Asterisks indicate statistically significant comparisons (P<0.05, t-test, N (neurons): $Fmr1^{+/-}$=15, $Fmr1^{-/-}$=23).
As demonstrated in Figure 3-17a and quantified in Figure 3-18, Fmr1-KO FS neurons fired with a significantly lower rheobase current than wild-type neurons. This was supported by altered input resistance (quantified in current clamp at close to resting potential as for excitatory neurons), which was found to be profoundly and significantly elevated in Fmr1-KOs (Wild-type vs. Fmr1-KO: 141±20 MΩ vs. 275±38MΩ, mean±SEM). As with excitatory neurons, no change to whole-cell capacitance was detected in Fmr1-KO FS neurons. Consequently, membrane time constants were nearly doubled in Fmr1-KO cells (Wild-type vs. Fmr1-KO: 18.1±2.6ms vs. 31.0±3.3ms, mean±SEM). Similar to excitatory neurons, AP half-widths were significantly broader at threshold in Fmr1-KO FS neurons, while no changes in height or shape of AHP were observed.

Dynamic changes in FS spike shape and rate during repetitive firing behaviour were compared between genotypes (see Figure 3-17 for example recordings) at threshold (Figure 3-18) as well as during sustained twice-rheobase current injections (Figure 3-19). Fmr1-KO FS neurons fired significantly fewer APs during a 500ms current step than wild-type neurons (Figure 3-19a). Additionally, instantaneous firing frequency was significantly slower in Fmr1-KOs both at the start of the evoked AP train and during steady-state firing (average of last two evoked APs), leading to a significant reduction in frequency accommodation. Changes to spike shapes during repetitive firing were observed in Fmr1-KO neurons (Figure 3-19b, also compare example phase plots in Figure 3-17). Similar to at threshold (Figure 3-18), AP height was unaltered between genotypes and was consistently maintained during the train and showed minimal
amplitude relaxation in either case. Conversely, Fmr1-KO FS neuron AP half-widths were significantly broader than in wild-type neurons for the duration of the firing response, with little time-dependent broadening observed for either genotypes. Finally, for the speed of the AP rising and falling phases, strong changes between genotypes were seen on the first AP, where both were initially significantly slower in Fmr1-KOs. During repetitive firing, both AP rising and falling rates underwent significant relaxation in wild-type neurons but those from Fmr1-KOs did not relax further. Consequently, AP rising and falling speeds converged between genotypes during steady state firing despite AP half-widths being maintained significantly broader.

Together these results demonstrate that, in stark contrast to previous findings from animals 3~4 days older, intrinsic properties of FS interneurons that support sustained high rates of firing are profoundly impaired in Fmr1-KOs at P10-11.

Interestingly, while aspects of intrinsic firing phenotypes appear common to layer 4 excitatory and FS inhibitory neurons, cell-type specialisations in firing behaviour could arise through independent disrupted control mechanisms in the absence of FMRP. For example, fractional elevations in neuronal input resistance in Fmr1-KOs were disproportionate between the two cell classes (~30% for excitatory neurons vs. ~50% for FS interneurons).
Figure 3-19 Spiking properties of Layer 4 Fast-Spiking (FS) interneurons during sustained AP firing induced by depolarisation with a 500ms twice-rheobase current injection.

a). Reduced rate of AP firing in Fmr1-/Y FS interneurons as analysed by total no. APs/500ms (left), Instantaneous frequency (1/inter-spike interval) of first and last two APs in train (centre), and instantaneous AP frequency as a function of spike number (right). Light colours indicate individual neurons, thick bars/lines are mean±SEM for each dataset. Asterisks on left and right graphs indicate parameters significantly different between genotypes compared by t-test (p<0.05, N (neurons): Fmr1+/Y =15, Fmr1-/Y =27). Asterisks on centre graph indicate significantly different frequencies between genotypes compared by one-way ANOVA with Bonferroni’s post-test for multiple comparisons (p<0.05, N: Fmr1+/Y =15, Fmr1-/Y =27).

b). Fmr1-/Y FS neuron spike shapes are altered on the first AP and undergo less adaptation during sustained firing. Statistical tests are ANOVAs and significance is reported as in a). N (neurons): Fmr1+/Y =15, Fmr1-/Y =27.
a

Instantaneous frequency

ANOVA first vs. SS:
+/Y: **, -/Y: n.s.

b

AP halfwidth (ms)

ANOVA first vs. SS:
+/Y: n.s., -/Y: n.s.

AP height (mV)

ANOVA first vs. SS:
+/Y: n.s., -/Y: n.s.

Depolarisation speed (Maximum, mV.ms⁻¹)

ANOVA first vs. SS:
+/Y: **, -/Y: n.s.

Repolarisation speed (Minimum, mV.ms⁻¹)

ANOVA first vs. SS:
+/Y: ***, -/Y: n.s.
Moreover, whereas both absolute values and genotype-dependent differences in AP rising speed were comparable for each cell type, AP falling speeds were nearly twice as fast in wild-type excitatory neurons compared to wild-type FS cells. Thus the deficit in *Fmr1-KOs* was disproportionately greater in mutant neurons. It is possible that this effect arises from differential susceptibilities of the cells’ individual ion channel components to loss of FMRP. For example, specialisations in FS interneurons include strong expression of 2-pore TASK (Taverna et al., 2005; Brickley et al., 2007; Goldberg et al., 2010) and *Kv3.1* channels (Du et al., 1996; Rudy and McBain, 2001; Macica et al., 2003), underlying their notably low input resistance and fast-spiking behaviour, respectively.

Whilst AP firing phenotypes of FS neurons in the present study are reminiscent of those following genetic, pharmacological or dynamic clamp ablation of channels, they are not as severe (*Kv3.1* channels: Joho et al. 1999; Lien and Jonas 2003; Espinosa et al. 2008; TASK channels: Goldberg et al. 2010)\(^{29}\). This is perhaps to be expected: for example, while FMRP co-immunoprecipitates with *Kv3.1* in synaptosomal preparations and its mRNA is a known FMRP target, no change in its protein expression or polysomal mRNA concentration are observed in the absence of FMRP (Darnell et al., 2001; Strumbos et al., 2010). This is consistent with the proposed role for FMRP in activity-dependent modulation of *Kv3.1* expression in a sub-population of channels rather than the total cellular cohort of channels (Strumbos et al., 2010).

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\(^{29}\) Interestingly, these studies additionally demonstrate alterations to thalamocortical oscillations *in vivo*, and depolarization induced *in vitro* oscillations.
3.5.10. Paired connectivity between identified neuronal classes in Layer 4

Older Fmr1-KO animals show specific reductions in motifs of excitatory-FS connectivity (Gibson et al., 2008; Patel et al., 2013). Moreover, altered ratios of inhibitory/excitatory spontaneous transmission onto layer 4 excitatory neurons were observed in the present study at P10-11 (Figure 3-12). Therefore the statistics of paired connectivity were investigated between these cell classes. Simultaneous patch recordings were made from visually identified FS and excitatory neurons (“FS-Ex. paired recordings”) within the same barrel in slices from Fmr1-KO or wild-type animals. Cell type was confirmed by AP firing pattern in response to 500ms depolarising steps before testing connections. Putative connections were tested in current-clamp by injecting brief (typically 10ms) steps of current into the presynaptic cell to elicit a single action potential (Figure 3-20a). Connections were identified as short-latency currents in recordings from postsynaptic cells (voltage clamped at -50mV or -70mV for testing GABAergic and glutamatergic connections, respectively) and were averaged across ten repeated trials at 0.01Hz. Here, in contrast to the specific unidirectional reduction found at P14 and P28 (Gibson et al., 2008), connection probability between FS and Ex. neurons was profoundly reduced by approximately 50% in each direction for P10-11 Fmr1-KO slices compared to those from wild-type littermates (Figure 3-20b).
Monosynaptic paired connectivity between Layer 4 FS and EX neurons is bidirectionally reduced in Fmr1<sup>−/−</sup> at P10-11.

**a).** Example recording configuration for testing a FS to EX paired connection. An FS neuron is depolarised to fire in current clamp with a brief (10ms, 2nA) current pulse. The putatively connected EX neuron is voltage-clamped at -50mV, providing 21mV (outward) driving force for a GABA<sub>A</sub> current. Monosynaptic connection is verified by the presence of a short (<2ms) latency IPSC in the EX cell. The reciprocal procedure is used to test the EX to FS connection, with the FS cell voltage clamped at -70mV (70mV of inward driving force for the glutamatergic current).

**b).** Connection probability between tested pairs of FS and EX cells. Asterisks indicate statistically significant differences (p<0.05, one-sided Chi-squared test).
Overall, a strong bidirectional reduction in recurrent connectivity between these cell types was observed: the probability of an FS-Ex neuron pair being reciprocally connected was reduced from 35% in wild-type slices to 14% in slices from *Fmr1*-KOs. This value remains within probabilistic expectations under the hypothesis of statistical independence for reciprocal connections, i.e. where the expected reciprocal connection probability is the product of the sampled incidences of unidirectional connections in each direction, as in Beierlein et al. (2009). Whilst in the present study in wild-type slices, experimentally determined connection probability in FS to Ex pairs was lower than that reported previously at P14 and P28 (Gibson et al., 2008), it was consistent with both a subsequent study from the Gibson laboratory (Patel et al., 2013) extended to P13-16 mice, as well as with a previous study from the Isaac laboratory at P9-11 (Chittajallu and Isaac, 2010). For *Fmr1*-KO recordings in the present study at P10-11, the reduced FS to Ex. connection probability was most consistent with that reported by Daw et al. 2007a for wild-type connections at P8-9 (Comparisons to findings from sensory-deprived animals in the literature will be made in subsequent chapters). In the literature at the current time, a comparable developmental series has not been performed in the barrel cortex for connectivity from Ex. to FS neurons, but connection probability in the present study was again lower than that previously reported at P14 and P28 (Gibson et al., 2008; Patel et al., 2013). Although highly variable, unitary current amplitudes between connected FS-Ex. pairs were not significantly different between genotypes for either direction (Figure 3-21a).

30 N.b. these references, the current study and Daw et al. 2007a all used congenic mice maintained on the C57Bl/6J background.
Figure 3-21 Dynamics of intracortical connections between FS and EX neurons in juvenile Layer 4.

**a-b.** Normal strength and onset latency of currents for connections in both directions between tested Fmr1<sup>+/Y</sup> (red) FS-EX pairs compared to those from Fmr1<sup>-/Y</sup> recordings (blue) (p>0.05, t-test, (N: FS to EX: Fmr1<sup>+/Y</sup> =9, Fmr1<sup>-/Y</sup> =8, EX to FS: Fmr1<sup>+/Y</sup> =9, Fmr1<sup>-/Y</sup> =6). N's indicate neurons

**c-d.** Short-term depression (STD) of connections between Fmr1<sup>-/Y</sup> EX and FS neurons is excessive in both directions. **c.** Example responses for a bidirectionally-connected Fmr1<sup>-/Y</sup> paired recording. Presynaptic cell was stimulated at 50Hz. Note the faster/stronger depression of postsynaptic currents in the EX to FS direction. **d.** Genotype average STD of intracortical currents, normalised to initial (steady-state) current amplitude. Bars show mean±SEM-normalised amplitude for each genotype. Solid lines and shaded regions show best fit±95% confidence intervals of a single exponential decay function to the data. For both connection directions, depression rates were significantly faster for connected Fmr1<sup>-/Y</sup> pairs and could not be explained by the same exponential fit (p<0.05, Extra sum-of-squares F-test, N: as in a-b).
Unitary current onset latencies (measured from time of presynaptic AP peak) were similarly unaffected (Figure 3-21b, similar results obtained for presynaptic AP peak to postsynaptic current peak times). Strikingly however, when connections were stimulated repetitively at 50Hz (e.g. Figure 3-21c), exaggerated short-term depression was observed for connections in both directions: Fmr1-KO connections displayed significantly faster and more progressive attenuation in postsynaptic current amplitude than those in wild-type slices (Figure 3-21d). Rates of depression at these synapses were asymmetric, as has been reported previously (Beierlein et al., 2009), showing stronger depression in the FS to Ex. direction. This effect is due to synapse-specific differences in presynaptic transmitter release probability, dictated in part by postsynaptic cell type-dependent expression of voltage-gated calcium channels at the presynaptic terminal (Ali and Nelson, 2006; Földy et al., 2007; Zaitsev et al., 2007; Tottene et al., 2009).
3.5.11. Spontaneous glutamatergic synaptic input to Fast-Spiking neurons is altered in Fmr1-KOs

In addition to inputs from local excitatory neurons, FS neurons in layer 4 receive potent excitatory thalamocortical inputs (Swadlow and Gusev, 2000; Porter et al., 2001; Daw et al., 2007a; Chittajallu and Isaac, 2010). These proportionally less numerous but potent synapses are distinct from intracortical inputs (Beierlein et al., 2009; Chittajallu et al., 2013) by physiological specialisations including Ca$^{2+}$-permeable AMPA receptors (Hull et al., 2009), clustered synaptic terminals (Bagnall et al., 2011) and higher release probability synapses (Gabernet et al., 2005; Cruikshank et al., 2010). Since strong reductions in connectivity between intracortical excitatory inputs to FS neurons were observed in the present study, it was envisioned that an easy in vitro prediction would be a strong reduction in the rate of spontaneous EPSCs of FS neurons would be observed in voltage clamp recordings from these cells. Indeed, PV$^{+ve}$ neurons appear to form dense, non-specific reciprocal connections with neighbouring excitatory cells with a probability that falls off with inter-somatic distance as a function of their axonal-dendritic overlap (Packer and Yuste, 2011; Levy and Reyes, 2012)$^{31}$, whereas thalamic innervation of Layer 4 FS (putatively PV$^{+ve}$ ) cells forms a relatively sparse but powerful minority of synaptic input (White, 1978; Bagnall et al., 2011). Counter to this hypothesis, rates of detected sEPSCs were nearly doubled on average in recordings from Fmr1-KO FS neurons compared to littermate neurons (Figure 3-22). Whilst no changes in central tendency of sEPSC amplitudes were observed between

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$^{31}$ This is not a general rule for neocortical interneurons however (e.g. for Som$^{+ve}$ cells: Fino and Yuste 2011; Chiu et al. 2013)
genotypes when cells were considered individually, population averaged mean distributions (see methods) were significantly different and steeper in the fourth quartile in Fmr1-KOs, suggesting a reduced contribution of large-amplitude sEPSCs. It is tempting to speculate that given their size, these events constitute synaptic release from thalamocortical terminals, known to be mediated by fast, Calcium-permeable GluA2-lacking AMPA receptors (Hull et al., 2009), it should be possible to parse sEPSCs by origin by comparing their size and kinetics. However, amplitudes of individual sEPSCs were found to be un- or weakly-correlated to their decay kinetics for all FS cells recorded (data not shown). Thus, further work is needed to describe changes at thalamocortical inputs to FS neurons in Fmr1-KOs.

Finally, a weak trend towards slower sEPSC decay was observed in Fmr1-KOs when the average sEPSC per cell was considered, suggesting that little change to sEPSC kinetics occurred (Figure 3-22c, right).

Together, these data show that synaptic inputs to FS interneurons are altered at P10-11 in the absence of FMRP, with a robust increase in the rate of spontaneous transmission observed in Fmr1-KOs. Since paired recordings explicitly demonstrated a strong reduction in intracortical excitatory synaptic input, a strong hypothesis to explain the increase in sEPSC frequency is an increase in number or release probability of thalamocortical synapses. A contribution from elevated release probability at intracortical synapses cannot be ruled out however, since short-term depression was stronger for Fmr1-KO Ex. to FS paired connections – given the magnitude of sEPSC rate increases in Fmr1-KO FS neurons however, it is unlikely that intracortical changes are the sole contribution to this effect.
Figure 3-22 Spontaneous glutamatergic inputs (sEPSCs) onto Layer 4 Fast-Spiking (FS) interneurons are smaller but occur at a higher rate in $Fmr1^{-Y}$ recordings (KMeSO4 internal solution, $V_{\text{hold}} = -70\text{mV}$).

**a).** Example voltage-clamp recordings (left) and all sEPSCs recorded during 60s aligned and averaged (centre) from $Fmr1^{+/Y}$ (blue) and $Fmr1^{-Y}$ (red) FS neurons.

**b).** Significantly more sEPSCs were recorded onto $Fmr1^{-Y}$ FS interneurons during 60s than onto those from littermates (p<0.02, Mann-Whitney, N (neurons): $Fmr1^{+/Y}$ =13, $Fmr1^{-Y}$ =19).

**c).** Cumulative distributions of amplitudes (left) and inter-event intervals (IEIs, centre) of sEPSC onto L4 FS interneurons recorded during a 60s window. Thin line are cumulative distributions of individual neurons, Thick lines show the bin-by-bin mean±SEM of bootstrap-resampled distributions in which each neuron contributes 20% of events recorded in 60s to population dataset. Mean amplitude and IEI distributions were significantly different between genotypes (K-S test, N: $Fmr1^{+/Y}$ = 13, $Fmr1^{-Y}$ = 19). Statistically significant differences are similarly obtained by the same tests if alternatively all events from each cell are pooled into a population distribution. Right: no statistically significant change between genotypes was observed for sEPSC decay kinetics (p>0.05, t-test, N (neurons): $Fmr1^{+/Y}$ =13, $Fmr1^{-Y}$ =19).
**Figure a**

- Graph showing traces of sEPSCs for different genotypes, with labels "Fmr1+/Y" and "Fmr1-/Y".
- Peak amplitudes: 50pA, time scale: 200ms/2ms.

**Figure b**

- Scatter plots with box plots showing:
  - Number of sEPSCs detected per 60s.
  - Cell mean decay tau (ms).

**Figure c**

- Cumulative fraction plots for sEPSC amplitude and inter-event interval, with asterisks indicating significance levels: *p<0.001.
3.6. Discussion

3.6.1. Altered intrinsic neuronal properties of Fmr1-KO
Layer 4 excitatory neurons are not consistent
with a general developmental delay

As previously discussed, the thalamocortical responses of cortical layer 4
neurons undergo a coordinated program of developmental maturation
during the first postnatal week (Daw et al., 2006, 2007a; Chittajallu and
Isaac, 2010) that is central to their ability to accurately respond to and
encode sensory information (Ghazanfar et al., 2000; Panzeri et al., 2001).
Whilst these studies have focused intensely on developmental changes to
synaptic physiology that occur during the critical period for
thalamocortical LTP (i.e. approx. P3-P7, Crair and Malenka 1995) and a
strong consensus has emerged that changes in synaptic properties drive
timing changes in neuronal firing (Isaac et al., 1997; Feldman et al., 1998;
Egger et al., 1999; Foeller and Feldman, 2004; Daw et al., 2007b; Hull et al.,
2009; Chittajallu and Isaac, 2010), changes in the intrinsic responsiveness to
synaptic stimuli by the postsynaptic Layer 4 neuron also contribute to this
process. Daw and colleagues demonstrated a developmental transition in
the intrinsic properties of Layer 4 neurons and have suggested that
interaction between the membrane properties of Layer 4 neurons and a
rapidly plastic postsynaptic glutamate receptor population is responsible
for developmental (and acute LTP) changes to firing precision (Daw et al.
In Layer 4 of the barrel cortex, the developmental epoch between the end of the critical period for thalamocortical LTP and the onset of patterned whisker exploration (postnatal days 7~12), is a period of ongoing activity-dependent circuit maturation (Daw et al., 2007a; Chittajallu and Isaac, 2010; Ashby and Isaac, 2011). During this developmental period, surprisingly little information is available in this model system about changes, activity-dependent or otherwise, to Layer 4 excitatory neuron intrinsic physiology.

In FS interneurons, short periods of whisker deprivation during the critical period for development of thalamocortical FFI (whisker trimming from P6-11) have no effect on intrinsic excitability despite large changes to synaptic innervation from sensory thalamus (Chittajallu and Isaac 2010b, supplemental materials). Conversely, longer periods of deprivation (up to P30) produce marked changes in FS neuron spiking characteristics, including spike waveforms and intrinsic membrane properties (Sun, 2009) in addition to changes in synaptic function.

Numerous developmental studies in primary sensory cortices of *Fmr1-KO* mice have raised the possibility of altered circuit maturation timing during early postnatal critical periods (Bureau et al., 2008; Bureau, 2009; Harlow et al., 2010; Meredith et al., 2012; Till et al., 2012; Gonçalves et al., 2013; Kim et al., 2013; Vislay et al., 2013). The extent to which circuits of the young FMRP-null cortex mimics that of sensory-deprived animals remains unclear and will be discussed with reference to data from the present study in later chapters. However, it is conceivable that changes to synaptic physiology resulting from loss of FMRP (e.g. Harlow et al. 2010) could trigger homeostatic changes to neuronal excitability in a manner
reminiscent to that shown to occur following developmentally restricted sensory input and acute plastic manipulations (Breton and Stuart, 2009; Nataraj et al., 2010; Nataraj and Turrigiano, 2011).\footnote{Although the majority of these studies focus on cortical L5 neurons, a minority has examined homeostatic regulation of intrinsic properties in L4 excitatory neurons, and all outwidth (older than) the ages discussed in the context of this thesis. For example, Bekisz and colleagues (Bekisz et al., 2010) find a BK-channel mediated change in excitability specific to excitatory neurons following classical conditioning paired with whisker stimulation, whereas Wang and colleagues find no intrinsic change in visual cortex following monocular deprivation (Wang et al. 2012, Figure 6).}

These ideas were explored in Section 3.3.4. Overall, this dataset suggests that P10-11 \textit{Fmr1-KO} excitatory neurons, as judged by their AP firing behaviour at threshold, are most consistent with age-matched wild-types despite showing a trend towards weaker distinction against younger wild-type neurons. Conversely, the intrinsic excitability of P10-11 \textit{Fmr1-KO} neurons (as judged by their rheobase, input resistance and membrane time constant) is significantly elevated with median values of population distributions most consistent with wild-type neurons up to six days younger. It should be noted however that confidently “ageing” a neuron by such physiological comparisons is extremely hard due to the small, scattered and apparently highly continuous\footnote{Hence the choice of potentially underpowered non-parametric statistical tests of ranked medians for these comparisons, to avoid making assumptions of population distributions.} developmental progressions that take place for wild-type neurons during this period of development. Furthermore, of this dataset, P10-11 \textit{Fmr1-KO} neurons showed the greatest degree of intra-animal and overall population scatter, but no change in distribution skew or kurtosis (data not shown). This suggests that a potential genotype-mediated change in intrinsic excitability between neurons from animals of the same age may be mediated by a continuous shift in the input resistance, but with increased variability between
sampled neurons, rather than a step-wise shift by of a sub-population of neurons.

3.6.2. Lack of resonant peaks in the Layer 4 Ex neuron intrinsic frequency response

Resonant frequency bands, visible as discrete peaks in impedance spectra, have been demonstrated in several cell types in diverse areas of the brain including hippocampus, entorhinal cortex and Layer 5 of primary sensory cortex. Resonance emerges due to interaction between intrinsic low-pass filter responses with high-pass and band-pass contributions from conductances as diverse as $I_{Kv}$, $I_h$, $I_{NMDA}$, persistent $I_{Na}$, and t-type $I_{Ca}$ (reviewed in Llinás 1988; Hutcheon and Yarom 2000). Specialised tuning of neurons by resonant intrinsic mechanisms serves to selectively amplify preferred input frequency bands (Schreiber et al., 2004), facilitating entrainment by oscillatory input (reviewed in Buzsáki 2010; Fell and Axmacher 2011).

Due to the small amplitudes of voltage deflections induced by these experiments, it is unlikely that significant contamination of impedance spectra by voltage-gated channel activity is present in this analysis. Such a non-linear inductive artefact would be expected to manifest as positive reactance when instead of considering the absolute magnitude of $Z(f)$, (i.e. $|Z(f)|$), the complex impedance is examined by plotting the imaginary component of $Z(f)$ (Reactance, $\Omega$) as a function of the real component (Resistance, $\Omega$) (e.g. Cole and Curtis 1936; Puil et al. 1986; Zemankovics et al. 2011). This behaviour was not observed for impedance spectra.
calculated in the present study, consistent with experimentally-derived purely passive impedance responses or those in which contributions from active currents are pharmacologically blocked (e.g. Puil et al. 1986, figure 4; Zemankovics et al. 2011, figures 4 and 6).

However, it is important to note that whilst pioneering in vivo recordings of awake behaving sub-threshold membrane potential dynamics by the Petersen laboratory demonstrate a cell-type and behavioural-state specific recruitment of barrel cortex Layer 2/3 neuronal to different frequencies (Crochet and Petersen, 2006; Crochet et al., 2011; Sachidhanandam et al., 2013), no evidence exists suggesting that the spike timing of layer 4 excitatory neurons possess a defined resonant frequency band (Llinás et al., 1991), or undergo theta modulation (Delacour et al. 1990, figure 3) despite being subject to theta-band input during sleep (Sirota et al., 2008). Conversely, high frequency (gamma) thalamocortically- and/or cortically-sustained circuit oscillations are present both spontaneously during developmental and during adulthood, where they are linked to sensory perception (Llinás et al., 1991; Oke et al., 2010; Minlebaev et al., 2011; Khazipov et al., 2013). The implications of developmentally disrupted circuit oscillations for FXS will be discussed alongside relevant data in Chapter 5.

34 Perhaps such a slow clocking signal would offer inferior and detrimental frequency resolution to a circuit optimised to detect correlations in ascending thalamocortical input with sub-millisecond precision – unlike (e.g.) in the olfactory system (Margrie and Schaefer, 2002) – or, temporally-binding signals may be provided by rhythmic motor feedback (Moore et al., 2013)
4. **Circuit-distributed abnormalities distort frequency-dependent gating of thalamocortical inputs in young *Fmr1-KO* barrel cortex**

4.3. Key findings:

1. The normal maturation of thalamocortical feed-forward inhibition is altered in *Fmr1-KOs* such that in P10-11 Ex. neurons:
   a) The distribution of FFI strengths received by Layer 4 excitatory neurons is affected: some receive no FFI, whilst the remainder receive stronger FFI on average. This increase is consistent with a relative increase in evoked FF-IPSC current amplitude.
   b) The synaptic kinetic of FFI are altered such that decay kinetics of FF-IPSCs are slower, and the delay between excitation and onset FFI is longer.
   c) Short-term depression at TC excitatory inputs and FF-inhibitory inputs to Layer 4 excitatory neurons is faster, stronger and more asymmetric in *Fmr1-KOs*, such that the run-down of FFI is faster and more sensitive to stimulation frequency during behaviourally realistic input trains.

2. Interactions between these electrophysiological abnormalities and their effects on FFI performance are explored through computational simulations, predicting the following changes in *Fmr1-KO* compared to wild-types:
   a) Neurons with no or weak FFI showed larger, slower EPSPs, and show excessive synaptic integration and voltage summation of low frequency inputs - capable of ectopically depolarising the model neuron to fire.
   b) Adding stronger FFI improved the temporal coincidence detection window for model inputs but could not correct hypersensitivity to high frequency stimulation in the *Fmr1-KO* model.
c) Attempting to “repair” the temporal sensitivity phenotype in silico showed that these effects arose from circuit-distributed phenotypes and provided insight into parameter sensitivity.

d) These simulations were largely predictive of real Ex. neuron responses to thalamocortical stimulation for single, paired and short burst stimulation of VB thalamus, as tested experimentally with current-clamp recordings.

3. Strongly exaggerated summation of TC inputs was found in Layer 4 Ex. neurons during burst stimulation of VB thalamus at high frequencies (20~50Hz).

4. Frequency gating performance of TC responses was impaired in Fmr1-KO slices: low frequency VB stimulation (5x pulses at 5~10Hz) could ectopically recruit intracortical network activity in recordings from both Ex. neurons and FS interneurons in L4.
4.4. Introduction

In the previous chapter, the intrinsic electrophysiological properties of Layer 4 excitatory neurons were found to be abnormal in brain slices from P10-11 *Fmr1*-KO mice. In particular, input resistance and membrane time constant were strikingly elevated and longer, respectively. Using increasingly biologically relevant single-cell manipulations (i.e. D.C. current injections, sinusoidal current waveforms and finally spontaneous synaptic inputs), it was demonstrated that these changes distort the representation of stimuli by affecting the magnitude and timing of both subthreshold and firing responses of *Fmr1*-KO neurons. How do these effects contribute to distorting the processing of sensory inputs?

As explored in the previous chapter, the high input resistance and slow membrane time constants intrinsic to Ex. neurons in Layer 4 lead to a pronounced low-pass filter effect on their current-voltage response transformation. This would suggest that they are not intrinsically optimised for discriminating input patterns by detecting correlations in their synaptic inputs. Indeed, model responses in this chapter suggest that this performance would be suboptimal if the voltage kinetics relied solely on the innate synaptic kinetics. Furthermore, the excessively long membrane time constants found in *Fmr1*-KO neurons in the current study are predicted to further slow the voltage response.

In this chapter, the developmental emergence of FFI is explored in *Fmr1*-KOs and is found to be abnormal in its strength, timing and use-dependent activation. The interaction between these and changes to postsynaptic
intrinsic properties reported in the previous chapter are explored using numerical simulations. These model results show that dysfunction in this circuit arises from multiple interacting phenotypes, and furthermore predict an altered window for temporal coincidence detection and a postsynaptic hypersensitivity to high frequency TC stimulation in the *Fmr1*-KOs, which is subsequently experimentally verified. Together, data presented in this chapter provide the first mechanistic description of abnormal circuit responsiveness to ascending thalamocortical inputs in the *Fmr1*-KO cortex, which is predicted to severely impact the processing of sensory information, and impact upon subsequent cortical development.

4.5. Results

4.5.1. Voltage-clamp characterisation of thalamocortical FFI

4.5.1.1. Normal developmental maturation of intracellular chloride ion concentration in *Fmr1*-KO Layer 4 excitatory neurons

In order to compare the strength of feed-forward inhibition in *Fmr1*-KO brain slices to those from wild-types, TC-evoked excitatory (EPSCs) and feed-forward inhibitory post-synaptic currents (FF-IPSCs) were electrically isolated by voltage clamping cells at -70mV or 0mV, equilibrium potentials for GABA$_A$ ("E$_{GABA_A}$") and NMDA/AMPA currents respectively. This experimental manipulation is possible as E$_{GABA_A}$ is enforced by known chloride ion gradients between internal (via the patch pipette) and external aCSF solutions. Whilst the derived balance of GABA to AMPA strength
(“G/A ratios”) are valid under these conditions in recorded neurons, unaccounted variability in internal [Cl⁻] among un-patched cells could lead to different ratios, rendering this comparison inaccurate. This is a particular concern for recordings from early postnatal neurons, since the direction of the transmembrane ionic [Cl⁻] gradient, established by opposing actions of NKCC1 and KCC2 chloride transporters is strongly developmentally regulated (reviewed in Blaesse et al. 2009). It was therefore necessary to confirm that $E_{\text{GABA}_A}$ undergoes a normal developmental shift from depolarising to hyperpolarising in $Fmr1$-KO neurons before considering the strength of evoked currents.

For this experiment, gramicidin perforated patch recordings (Figure 4-1, methods) were chosen as an approach that minimally influences cytosolic chloride concentration. $E_{\text{GABA}_A}$ was measured in neurons from mice aged between P6 – P11 (Figure 4-1d). These results recapitulated findings of Daw et al. 2007, showing a significant trend towards more hyperpolarised values with age. Additionally, inter-animal variability reduced with age. At P10-11, mean values were not significantly different between genotypes (wild-type: $-62.3\pm4.3\text{mV}$, $Fmr1$-KO: $-62.7\pm3.3$).

While mean $E_{\text{GABA}_A}$ was more depolarised in the present study, comparable variability to that reported in Daw et al. (2007) was observed, highlighting the string inter-cell variability. The discrepancy in mean $E_{\text{GABA}_A}$ may arise through technical (including mouse strain) differences as well as greater sample sizes in the current study. Identical recording solutions were used; furthermore neither this nor the referenced study corrected for junction potential.
Figure 4-1 Maturation of intracellular chloride concentration follows normal developmental time-course during the second postnatal week in in *Fmr1*/*Y* Layer 4 neurons

a). Schematic recording configuration, showing gramicidin perforated-patch recording of a Layer 4 excitatory neuron and bulk stimulation of local pharmacologically-isolated GABAergic inputs. b). Example traces showing a typical perforated patch recording in which stable series resistance was obtained after ~15 minutes and could be maintained >30 minutes without patch rupture. Inset: example outward GABAergic current evoked by local bulk stimulation that was completely abolished in the presence of Picrotoxin. c). Example I/V plot showing peak evoked GABAergic current for a P11 *Fmr1*/*Y* neuron, averaged in depolarising and hyperpolarising directions at holding potentials between -90mV and -10mV. Black line shows linear best fit. Inset: highlighting stability of recordings over time; single trial responses at holding potentials of -80,-70,-60 and -50mV, repeated once at 20 and once at 40 minutes after forming seal. d). Day-by-day developmental progression of E_{GABA_a} between P6 and P11 for *Fmr1*/*Y* (blue) and *Fmr1*/*Y* (red) neurons. E_{GABA_a} for neurons of both genotypes was significantly more hyperpolarised at P10-11 compared to *Fmr1*/*Y* neurons at P7-8. No other pair-wise comparisons were significantly different, including P8-9 *Fmr1*/*Y* vs. P8-9 *Fmr1*/*Y*, or P10-11 *Fmr1*/*Y* vs. P10-11 *Fmr1*/*Y* (two-way ANOVA with Tukey’s honest significance test for multiple comparisons, Individual points are neurons (max. 2 per animal), bars are mean±SEM).
Resting membrane potential was similarly unaffected by genotype (Figure 3-3b) and effectively overlaid values for \( E_{GABA_a} \) (Figure 4-1d). This suggests that mean Cl\(^{-}\) driving force is minimal at mean (slice) resting potential, such that GABAergic input is likely to provide predominantly shunting rather than hyperpolarising inhibition under quiescent conditions, whilst providing strong hyperpolarising inhibition during network-driven GABAergic input, where cells are typically depolarised by ~15mV. However, the considerable variability of both measures could support different effects depending on within-cell values. \( E_{GABA_a} \) measurements presented in this section were obtained from different neurons to those in which resting potential was measured, thus it is impossible to exclude the possibility that electrochemical driving forces between \( E_{GABA_a} \) and resting potential may vary differentially between individual neurons both within and between genotypes. This was unsuccessfully addressed experimentally (due to difficulties in isolating resting potential \( E_{GABA_a} \) in individual Layer 4 excitatory neurons - and see Methods, Section 2.2.4). Overall, these results show that the Cl\(^{-}\) driving force is not significantly different when compared across genotype mean values. Therefore for the following experiments, in which \( E_{GABA_a} \) was standardised at -71mV for best comparison against previously published studies, uneven discrepancies in chloride driving force between wild-type and \( Fmr1\)-KO neurons can largely be ruled out in the current study.
4.5.1.2. The strength of thalamocortical feed-forward inhibition is abnormal in P10-11 Layer 4 Fmr1-KO barrel cortex

Whole-cell recordings were made *ex vivo* from voltage-clamped layer 4 excitatory neurons in slices prepared from P10-P11 mice whilst electrically stimulating TC afferents exiting VB (Figure 4-2). To internally control for inter-slice variability, the strength of FFI received by postsynaptic neurons was quantified as the peak amplitude ratio between FF-IPSCs and EPSCs (GABA:AMPA ratio, “G/A”, Figure 4-2b).

**Figure 4-2** Disrupted strength of thalamocortical feed-forward inhibition in young Fmr1<sup>+/Y</sup> Layer 4 excitatory neurons

**a).** Cartoon showing experimental configuration. Bulk thalamocortical afferents leaving VB thalamus are stimulated with a bipolar electrode (yellow lightening bolt). In a whole-cell recording from a Layer 4 excitatory neuron, direct thalamocortical EPSCs (1) and FF-IPSCs (2) can be isolated by voltage clamping voltage clamped by holding the neuron at E<sub>GABAa</sub> (-70mV) and E<sub>NMDA/AMPA</sub> (0mV) respectively. The strength of TC FFI (“G/A ratio” is quantified as the ratio of peak evoked current amplitudes.

**b).** Strength of TC FFI in P10-11 Fmr1<sup>+/Y</sup> (blue) and Fmr1<sup>−/Y</sup> (red) neurons. Bottom: Points indicate neurons, bars are mean±SEM. Unlike for Fmr1<sup>+/Y</sup> recordings (28 neurons, maximum of 3 neurons from each animal), in Fmr1<sup>−/Y</sup> neurons, some cells (8/38 neurons, maximum of 3 from each animal) were found to have no detectable FFI; light red bars indicate the mean±SEM strength of the remaining population of neurons (i.e. cells with G/A>0, hollow markers). Including Fmr1<sup>−/Y</sup> neurons with G/A=0, average strength of FFI was not significantly different to that of Fmr1<sup>+/Y</sup>, but excluding these neurons, (dark red bars and solid markers), the average strength was elevated over that of FFI in wild-type recordings (Fmr1<sup>+/Y</sup> vs. all Fmr1<sup>−/Y</sup> neurons: p=0.40, Fmr1<sup>+/Y</sup> vs. Fmr1<sup>−/Y</sup> neurons with G/A>0: p=0.005, Mann-Whitney.

Top: Cumulative histograms of FFI strength population distributions described above. Individually, all three distributions failed K-S tests for normal distribution (p<0.05). Compared to the distribution of Fmr1<sup>+/Y</sup> FFI strengths, the total Fmr1<sup>−/Y</sup> distribution was not significantly different (p>0.05, K-S test, N’s as described above). However, the surrogate Fmr1<sup>−/Y</sup> population excluding cells with no FFI was significantly different to the Fmr1<sup>−/Y</sup> distribution (p=0.001, K-S test, N’s as described above).
a

\[
\text{G/A} = \frac{\text{FF-IPSC}}{\text{EPSC}}
\]

b

Cum. Fraction (G/A ratio) vs. G/A ratio

* \( p < 0.001 \)
All tested wild-type neurons received FFI, with the majority (61%) receiving moderate strengths (G/A=0.5-4). In *Fmr1*-KO cells however, two changes to the distribution of recorded FFI strengths were observed. Firstly, 21% of cells received no FFI (G/A=0, 8/38 neurons, c.f. 0/28 wild-type neurons). Secondly, whilst the population distribution of FFI strengths including these neurons was not significantly different to wild-types, the remainder showed significantly stronger G/A ratios, such that mean FFI strength (withholding cells with G/A=0) was significantly elevated in *Fmr1*-KO neurons.

A similar increasing trend was observed when considering the ratio of Inhibitory to Excitatory charge transferred (i.e. when voltage clamping at 0mV vs. -70mV) during a 50ms window from the EPSC onset (Figure 4-3b). This measure is considered less precise since multiple staggered currents potentially resulting from stimulation of compound thalamocortical fibres would be a greater source of error during the decay phase compared to the current peak. Additionally, despite the voltage clamp at -70mV, variability in the small contribution of NMDA would be greater in this longer window, compared to at the current peak - dominated by AMPA currents.

As discussed below, NMDA:AMPA ratios at TC synapses onto *Fmr1*-KO L4 excitatory neurons were not explicitly tested in this study, but have been previously been shown to be unaltered compared to wild-type neurons at P10 using mice on the same background (Harlow et al., 2010). However, the Mg²⁺-insensitive NMDA component could represent a source of unaccounted experimental variability in Harlow et al. (2010).
Overall therefore, the distribution of FFI strengths onto Layer 4 neurons
*Fmr1-KO* is altered at this age such that some neurons (ectopically) receive
no FFI, whilst the strength of FFI received by the remainder of the
population is elevated.

When comparing evoked EPSCs and FF-IPSC strengths individually, for a
given EPSC strength, significantly increased FF-IPSC amplitude was
observed in *Fmr1-KOs* (Figure 4-3a). A similar trend was observed when
expanding the G/A ratio as a function of charge transferred during a 50ms
post-stimulus window (Figure 4-3b). This suggests that stronger FFI
occurred as a result of stronger evoked GABAergic currents rather than a
reduction in TC-evoked EPSC amplitude. Indeed, when evoked EPSCs and
FF-IPSCs were considered separately as individual population mean
values, FF-IPSCs were significantly stronger both in amplitude and charge,
whereas evoked EPSCs were not significantly different, despite showing a
trend towards being weaker by both measures (thalamic stimulation
strength was not significantly different between genotypes (data not
shown)).

**4.5.1.3. Altered timing and synaptic kinetics of TC FFI in Fmr1-KOs**

Widespread timing changes to the thalamocortical FFI circuit were found
in *Fmr1-KO* recordings in the present study (Figure 4-4 and 5). Firstly, the
mean lag between the onset of TC-evoked EPSCs and the arrival of FF-
IPSCs was significantly longer by ~0.5ms (~120%) in *Fmr1-KO* neurons
compared to those from littermates (Figure 4-4b bottom).
Figure 4-3 An increase in the relative strength of feed-forward GABAergic input strength increases the strength of thalamocortical feed-forward inhibition in young Fmr1<sup>-/-</sup> Layer 4 excitatory neurons.

a). G/A ratio expressed as peak amplitude. Right: data replotted from Figure 4-2b, again excluding Fmr1<sup>-/-</sup> neurons with no FFI (N’s and descriptive statistics as in Figure 4-2b). Left, FF-IPSC amplitudes as a function of evoked TC amplitude. Points indicate individual neurons, bars are mean±SEM. Evoked EPSC amplitudes were not significantly different between genotypes (p>0.05, Mann-Whitney, N: Fmr1<sup>+/+</sup> = 28, Fmr1<sup>-/-</sup> = 30; results unchanged when Fmr1<sup>-/-</sup> neurons with G/A=0 are included (i.e. Fmr1<sup>-/-</sup> = 38). The average FF-IPSC amplitude was significantly increased in Fmr1<sup>-/-</sup> neurons for these recordings compared to littermates (p<0.05, Mann-Whitney, N’s as above). Fitted lines indicate best-fit linear trends to evoked current amplitude ratios, through the origin. Dotted line indicates unity. The best-fit FFI amplitude ratio trend was significantly elevated for Fmr1<sup>-/-</sup> recordings compared to Fmr1<sup>+/+</sup> recordings (Extra-sum-of-squares F test, p<0.0001, F=34.95).

b). G/A ratio expressed alternatively as a function of charge transferred in a 50ms window after the onset of the TC EPSC. Right: data from Figure 4-2b, replotted as ratio of charge transferred, again excluding Fmr1<sup>-/-</sup> neurons with no FFI (N’s and descriptive statistics as in Figure 4-2b): no significant difference (p=0.10) was observed between genotypes. Left: as in a), but plotting charge transferred by individual current components. Average charge transferred by EPSCs was not significantly different between genotypes (p>0.05, Mann-Whitney, N: Fmr1<sup>+/+</sup> = 28, Fmr1<sup>-/-</sup> = 30; results unchanged when Fmr1<sup>-/-</sup> neurons with G/A=0 are included (i.e. Fmr1<sup>-/-</sup> = 38). Charge transferred by the average FF-IPSC was significantly increased in Fmr1<sup>-/-</sup> neurons for these recordings compared to littermates (p=0.045, Mann-Whitney, N’s as above). The best-fit FFI charge ratio trend was significantly elevated for Fmr1<sup>-/-</sup> recordings compared to Fmr1<sup>+/+</sup> recordings (Extra-sum-of-squares F test, p=0.0013, F=13.59)
a  Peak amplitude

b  Charge transferred
**Figure 4-4** Slower synaptic current onset and broader excitatory-inhibitory window during thalamocortical feed-forward inhibition in *Fmr1-KO* Layer 4

a). Example TC-evoked currents in voltage-clamp recordings from *Fmr1<sup>+/Y</sup>* (blue) and *Fmr1<sup>+/Y</sup>* (red), EPSCs and FF-IPSCs are shown individually scaled to peak amplitudes. Note the pronounced increase in decay time constant for *Fmr1<sup>+/Y</sup>* FF-IPSCs. Inset: time-base shown magnified to highlight the increased lag between EPSC onset and FF-IPSC onset.

b). Top: Mean EPSC and FF-IPSC post-stimulus onsets were significantly delayed in *Fmr1<sup>+/Y</sup>* recordings compared to in those from littermates. Points are individual neurons; bars are mean±SEM (EPSCs: 3.37±0.16ms (*Fmr1<sup>+/Y</sup>, N=34 neurons) vs. 5.5±0.22 (*Fmr1<sup>+/Y</sup>, N=36 Neurons), p<0.0001, t-test). (FF-IPSCs: 4.95±0.19ms (*Fmr1<sup>+/Y</sup>, N=34 neurons) vs. 5.9±0.20 (*Fmr1<sup>+/Y</sup>, N=36 Neurons), p<0.0009, t-test). Bottom: Mean EPSC - FF-IPSC onset lags for individual neurons were significantly slower in *Fmr1<sup>+/Y</sup>* recordings (1.55±0.11ms (*Fmr1<sup>+/Y</sup>, N=34 neurons) vs. 1.84±0.10 (*Fmr1<sup>+/Y</sup>, N=36 Neurons), p=0.048, t-test).
This broadened window between the arrival of excitation and inhibition is predicted to reduce the potency of elevated FFI strength in Fmr1-KO mice by permitting greater integration of excitatory synaptic charge transferred by TC inputs in L4 excitatory neurons before curtailment at the onset hyperpolarising FFI. Since unitary conduction time was not affected in paired recordings between FS interneurons and Ex neurons in Fmr1-KOs (Figure 3-21b), and Fmr1-KO FS cells displayed significantly slower $\tau_{\text{membrane}}$ (Figure 3-18), it is likely that this delay was introduced by slower integration of TC input charge in FS neurons, leading to slightly delayed AP firing in these cells. Interestingly, post-stimulus onset latencies for TC-evoked EPSCs were significantly slower in Fmr1-KOs excitatory neurons compared to littermates (Figure 4-4b top). Furthermore, TC-evoked EPSCs recorded in L4 FS interneurons similarly showed significantly delayed onset latencies in Fmr1-KOs (wild-type: 3.37±0.18ms N=12 animals vs. Fmr1-KO: 4.42±0.21ms N=9 animals, *p=0.013, t-test), raising the possibility that thalamocortical axonal anatomy or conduction velocity may be altered in Fmr1-KO animals at this age.

Secondly, rise and decay kinetics of FF-IPSCs were significantly slower in Fmr1-KO recordings, as measured by 20-80% rise time and single exponential decay time constants, respectively (Figure 4-5). A similar trend towards slower onset and decay kinetics was observed for Fmr1-KO TC-evoked EPSCs.
Figure 4-5 Slower synaptic kinetics of feed-forward inhibitory currents in recordings from Fmr1-KO Layer 4 excitatory neurons.

Rise time, decay time-constant (single exponential term) and half-width of TC-evoked EPSCs (a) and FF-IPSCs (b). Timing is summarised below (t-tests, statistically different findings denote p=0.05 significance level).

**EPSCs** N=23 (Fmr1+/Y) and N= 26 (Fmr1−/Y) neurons

<table>
<thead>
<tr>
<th>Timing (ms)</th>
<th>Fmr1+/Y (mean±SEM)</th>
<th>Fmr1−/Y (mean±SEM)</th>
<th>p value (t-test)</th>
</tr>
</thead>
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<tr>
<td>Rise time</td>
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<td>0.9±0.1</td>
<td>0.08</td>
</tr>
<tr>
<td>Half-width</td>
<td>2.6±0.3</td>
<td>3.3±0.4</td>
<td>0.23</td>
</tr>
<tr>
<td>Decay time-constant</td>
<td>2.8±0.2</td>
<td>3.1±0.3</td>
<td>0.41</td>
</tr>
</tbody>
</table>

**FF-IPSCs** N=27 (Fmr1+/Y) and N= 28 (Fmr1−/Y) neurons

<table>
<thead>
<tr>
<th>Timing (ms)</th>
<th>Fmr1+/Y (mean±SEM)</th>
<th>Fmr1−/Y (mean±SEM)</th>
<th>p value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rise time</td>
<td>0.7±0.1</td>
<td>0.9±0.1</td>
<td>*0.046</td>
</tr>
<tr>
<td>Half-width</td>
<td>7.4±0.5</td>
<td>9.2±0.6</td>
<td>*0.022</td>
</tr>
<tr>
<td>Decay time-constant</td>
<td>9.5±0.9</td>
<td>12±0.8</td>
<td>*0.035</td>
</tr>
</tbody>
</table>
4.5.1.4. Altered input frequency sensitivity of FFI in young 

_Fmr1-KOs_

FFI greatly improves the frequency resolution performance of synaptic circuits by curtailing the integration window for summing excitatory postsynaptic potentials (EPSPs). At TC inputs to L4 Ex neurons, which have a pronounced intrinsic low-pass filter response dictated by their long membrane time constants, discrimination between subtly different patterns of fast synaptic input presented during sensory input relies upon additional high-pass filtering imposed by FFI. The overall frequency transform performed upon TC inputs by L4 Ex neurons is thus a compound response of intrinsic properties augmented by the circuit-distributed effects of FFI.

Repetitive stimulation of high-release probability synapses causes use dependent rundown of neurotransmitter release, resolved as short-term depression (STD) of postsynaptic currents. In whole-cell recordings from _Fmr1-KO_ excitatory neurons, STD of TC-evoked EPSCs and FF-IPSCs was found to be faster, more progressive and showed greater asymmetry compared to that of littermates (Figure 4-6 to 8). Figure 4-6a shows example voltage-clamp recordings from wildtype and _Fmr1-KO_ neurons, isolating TC-evoked EPSC and FF-IPSC responses during five regular stimuli at 10Hz. Note the exaggerated rate of STD in the _Fmr1-KO_ example, for both EPSCs and FF-IPSCs. Furthermore, note the exaggerated asymmetry in the rate of rundown of the two current components relative to the wild-type example, such that FF-IPSC amplitudes were severely attenuated in the _Fmr1-KO_ trace after the first stimulus and completely
abolished after the second, compared to a more gradual depletion in the wild-type example. Conversely for direct TC EPSCs, whilst the wild-type example shows gradual attenuation of responses during the stimulus train, a strong paired-pulse depression effect was observed in the *Fmr1-KO* example, with little additional depression observed during subsequent responses. Short-term depression of EPSCs and FF-IPSCs evoked by 5x10Hz stimulation are compared (normalised to the steady-state current amplitude) for L4 Ex neuron recordings from 8 wild-type and 13 *Fmr1-KO* animals in Figure 4-6b. Significant differences between genotypes emerged after the third stimulus for EPSCs, and specifically on the second stimulus for FF-IPSCs. The dynamic depression of EPSCs and FF-IPSCs during repetitive stimulation was well captured by bi-exponential decay functions (shaded regions show best fit and ±95% confidence intervals). For both EPSCs and FF-IPSCs, fit parameters were significantly different between genotypes such that a shared fit could not adequately explain both datasets.

To explore the dynamics of FFI across thalamocortical firing frequencies known from previous thalamic recording studies to be involved in sensory coding during both quiet wakefulness and active whisker exploration (e.g. Poulet et al. 2012), VB was stimulated with short regular bursts: 5x5Hz to 5x50Hz, i.e. five stimuli at between 5-50Hz (Figure 4-7). Both EPSCs and FF-IPSCs depressed at faster rates in *Fmr1-KO* recordings relative to wild-type responses at all frequencies tested (Figure 4-7a). Additionally, considered independently at each frequency, normalised current responses to the second and fifth stimuli relative to initial steady-state responses were
either significantly different between genotypes or showed strong trends towards reduction in Fmr1-KOs, most notably for EPSCs (Figure 4-7c).

Best fits to FF-IPSCs were divided by those to EPSCs to derive the average frequency-dependent reduction in FFI strength during repetitive stimulation (Figure 4-7b, replotted to highlight frequency sensitivity in Figure 4-8). After the second stimulus at 5Hz, FFI onto wild-type neurons showed a ~13% mean strength reduction, which increased to ~42% with 50Hz stimulation. FFI onto Fmr1-KO neurons showed greater sensitivity to low frequencies, initially depressing by ~33% at 5Hz stimulation, progressing to ~53% after the second stimulus at 50Hz. During repetitive TC stimulation, the rate of FFI reduction was further affected in Fmr1-KOs, depressing faster and more progressively. Greater frequency-sensitivity was observed for depression of FFI in Fmr1-KOs, showing an extra 41% depression relative to wild-type responses after 5x50Hz stimulation, compared to an extra ~18% depression at after 5x5Hz stimulation.
Figure 4-6 Short-term depression of thalamocortical and feed-forward GABAergic inputs is faster and more progressive in Fmr1^{-/Y} neurons.

a). Example voltage-clamp recordings from a P11 Fmr1^{+/Y} neuron (top, blue) and a P11 Fmr1^{-/Y} neuron (bottom, red), showing short-term depression of EPSCs and FF-IPSCs during repetitive TC stimulation at 10Hz. Note the pronounced increase in rate and asymmetry of depression between EPSCs and FF-IPSCs in the Fmr1^{-/Y} example.

b). Short-term depression of EPSCs and FF-IPSCs during 5x10Hz stimulation: Quantification of evoked current amplitudes as a fraction of initial (steady-state) current amplitude. Points and solid error bars indicate mean±SEM normalized current amplitude after for each stimulus for Fmr1^{+/Y} (blue, N=8 (EPSCs) and N=6 (FF-IPSCs) neurons) and Fmr1^{-/Y} (red, N=13 (EPSCs) and N=10 (FF-IPSCs) neurons). Asterisks indicate individual stimulus responses that were significantly different between genotypes (t-test, p<0.05). Shaded regions are best±95% C.I. fits to bi-exponential decay functions (see methods). For both EPSCs and FF-IPSCs, the rate of depression for Fmr1^{-/Y} responses was faster and a single (i.e. shared) fit could not adequately explain the behaviour of both genotypes (Extra sum-of-squares F-test, EPSCs: p<0.0001, F=12.9, IPSCs: p=0.0006, F=8.17, N's as above).
Figure 4-7 Short-term depression of thalamocortical EPSCs and feed-forward IPSCs is excessive in P10-11 Fmr1-KO recordings across a range of behaviourally relevant stimulation frequencies.

a). Analysis as for Figure 4-6b, but extended for TC stimulation frequencies between 5-50Hz. Fit parameters (exponential rate constant and plateau amplitude) were significantly different between Fmr1+/− and wild-type littermates for all stimulation frequencies tested for both EPSCs and FF-IPSCs (P<0.001, extra sum-of-squares F-test, N (neurons) = Fmr1+/−: 8-19 (EPSCs), 6-12 (FF-IPSCs); Fmr1−/−: 13-14 (EPSCs), 10-13 (FF-IPSCs), varying according to stimulation frequency experiments).

b). Genotype-dependent average depression rate of FFI as a function of TC stimulation frequency, obtained by dividing FF-IPSC/EPSC population best fits in a). – Highlighting the faster, stronger and more frequency sensitive depression of FFI in Fmr1−/− neurons compared to that of littermates.

c). Normalised current amplitudes compared between genotypes for the second and fifth responses during TC burst stimulation at frequencies between 5-50Hz. Asterisks indicate statistically significant differences (p<0.05) between genotypes as a function of stimulation frequency (t-test, N's as for a).
a  
Stimulus: 5x5Hz  5x10Hz  5x20Hz  5x50Hz

Normalized mean peak current

Stimulus time (ms)

Normalized change in initial In/Ex ratio

b

Stimulus time (ms)

C

2nd stimulus  5th stimulus

Normalized Amplitude

Stimulation Frequency (Hz)
Figure 4-8 Exaggerated asymmetric short-term depression leads to faster and more progressive run-down of feed-forward inhibition in Fmr1-KO recordings.

Data in Figure 4-7b replotted on the same time-base to highlight average rate of FFI depression as a function of stimulus number at different stimulation frequencies, shown for Fmr1+/Y (left, blue) and Fmr1−/Y (right, red) recordings. Grey crosses indicate individual stimulus times, coloured crosses highlight 5x10Hz simulation, as shown by example voltage-clamp traces in Figure 4-6a.
4.5.2. Simulating the performance of TC FFI in *Fmr1-KO* Layer 4

In the above whole-cell voltage clamp recordings from *Fmr1-KO* neurons, changes were found to multiple parameters that have previously been demonstrated to sculpt the coincidence detection behaviour at TC inputs to L4. How do these individual circuit abnormalities interact to affect functional performance of the circuit by modulating temporal resolution of TC input detection? Such an interaction is likely to be complex and dynamic: Conceptually, the boosted intrinsic excitability and longer intrinsic time constants of Ex neurons are predicted to interact with both the longer Ex-In delays and onset kinetics for FFI responses and faster rate of depression FFI during repetitive TC activation to aid voltage summation of TC inputs at lower frequencies. Conversely the markedly longer decay kinetics of FF-IPSCs, and the shift towards stronger steady-state FFI tone observed in the present study would be predicted to work synergistically to enhance the efficacy of FFI by prevent excessive voltage summation.

To systematically disentangle these complex phenotypic interactions, a reduced circuit model was adopted. In particular, this approach has previously been used successfully to gain mechanistic insight into interactions among circuit/synaptic and cell-intrinsic properties; notably for the robustness of circuit outputs in the face of widespread natural variation in intrinsic firing properties (Grashow et al., 2010), and circuit-level homeostatic compensation for reduced intrinsic excitability in an experimental model of Autism (Walcott et al., 2011).
Voltage-clamp recordings were recreated \textit{in silico} with a simple computational model of interacting TC EPSCs and FF-IPSCs in a passive (leaky integrating, without active conductances, or “spiking” behaviour) somatic compartment. To explore the temporal resolution of the model somatic compartment, the voltage summation behaviour was monitored in response to different patterns of simulated synaptic input, whilst systematically varying the strength of FFI by increasing peak FF-IPSC conductance relative to that of EPSCs (Figure 4-9). To reduce the model complexity, Wild-type and \textit{Fmr1-KO} phenotypes were additionally grouped into four parameter supergroups: namely “passive properties” (input resistance, whole-cell capacitance and resulting intrinsic time constant), “synaptic kinetics” (rise time and decay kinetics of TC EPSCs and FF-IPSCs), “Ex-In window” (EPSC – FF-IPSC onset lag) and “short-term plasticity” (frequency-sensitive run-down of TC EPSCs and FF-IPSCs).

4.5.2.1. Simulated FFI (1): Circuit-distributed physiological changes affect the shape of thalamocortical EPSPs in young \textit{Fmr1-KOs}

When responses to a single model input were examined for wild-type parameters (“Full WT model”, blue), (Figure 4-10), in the absence of FFI, a rounded EPSP could be evoked from a steady-state “resting” potential of -60mV. Sequential strengthening of IPSCs to produce G/A ratios between 0.5~10 led to the emergence of EPSP-IPSP sequences. As expected (Daw et al., 2007a; Chittajallu and Isaac, 2010), addition of modest strength FFI (G/A ratios 0.5~2) rapidly changed the temporal kinetics of the EPSP, leading to a reduction in both the amplitude, by up to 60%, and duration.
(measured at half-height: “EPSP half-width”), from ~50ms to <10ms (Figure 4-11). With addition of FFI stronger than G/A ~2, negligible further reduction to the EPSP amplitude could be obtained, whilst progressive curtailment of EPSP duration continued at FFI ranges up to G/A ~3, subsequently becoming asymptotic towards ~1ms with higher G/A ratios. These simulations demonstrate the powerful effect of modest, physiological strength FFI on controlling the voltage response to excitatory input, capable of dramatically reducing naïve EPSP amplitudes by over half, and shortening their duration by more than an order of magnitude to attain responses with ~1ms kinetics. Interestingly, the temporal performance of the simulated voltage kinetics far exceeds those of either the excitatory or inhibitory currents individually; suggesting that precise timing of the EPSC-IPSC sequence is a crucial parameter of this effect.

To explore the consequences of individual Fmr1-KO phenotypes from slice physiology data on the EPSP kinetics, mean parameters from Wild-type recordings were systematically replaced with those from Fmr1-KO recordings (“WT with KO passive properties”, “WT with KO synaptic kinetics”, “WT with KO E-I delay”; coloured orange, green and teal, respectively in Figure 4-11). The effects are summarised as followed:

**Wild-type with Fmr1-KO intrinsic excitability** A powerful contribution of the (elevated) altered intrinsic excitability was observed in the absence of FFI, with amplitudes of uninhibited EPSPs larger by 20%, and longer by 10ms. With addition of modest strength FFI, these disparities diminished and were indistinguishable from the wild-type simulation at G/A >2. It was therefore hypothesized that stronger FFI onto L4 EX neurons, indicated by
elevated G/A ratios observed in *Fmr1*-KO recordings could be beneficial for the performance of TC EPSPs under conditions of elevated postsynaptic intrinsic excitability in this genotype.

**Wild-type with *Fmr1*-KO synaptic kinetics** The effects of the longer synaptic kinetics (dominated by slower FF-IPSC decay kinetics) caused further divergent effects on FFI performance: Without FFI, a slightly larger EPSP amplitude was observed when *Fmr1*-KO replaced wild-type kinetics, presumably due to a modest increase in excitatory charge transferred during the longer EPSC decay. Due to slower FF-IPSC kinetics (notably, longer decay constant), both amplitude and duration of EPSPs were hypersensitive to small increases in FFI, with progressive over-compensation (reduced amplitude) observed for strong FFI (G/A>2).

**Wild-type with *Fmr1*-KO EPSC-IPSC lag** The effect of ~0.5ms increased EPSC-IPSC lag was not observable for FFI ranges <2, thereafter strongly contributing to exaggerated EPSP depolarisation that persisted regardless of increased FFI strength, confirming that greater excitatory charge was always integrated, unaffected by FFI due to its slower onset.

**Full *Fmr1*-KO simulation** Finally, when combined together to reconstruct the complete *Fmr1*-KO response (“Full KO model”, red in Figure 4-11), a complex phenotypic interaction emerged whereby with no/weak FFI, elevated intrinsic excitability dominated, producing 120% larger and longer EPSPs. Simulated *Fmr1*-KO EPSPs were hypersensitive to modest increases in FFI (an effect dominated by synaptic kinetics for G/A ranges between 0.5~2), with EPSPs becoming shorter, albeit still larger in amplitude,
relative to wild-type simulations. Further increases in FFI strength (G/A<2) were ineffective at further constraining EPSP shape, with amplitudes and durations always exceeding those of wild-type simulations even for very strong (physiologically outlying) strengths of FFI (G/A=10).

4.5.2.2. Simulated FFI (2): Abnormal dynamic control of synaptic integration by FFI alters temporal resolution in the Fmr1-KO model

How does the effect of exaggerated frequency-dependent run-down of FFI in Fmr1-KO recordings alter the frequency discrimination behaviour of the model under simulated physiological input patterns? A simple phenomenological model of short-term plasticity (Varela et al., 1997, 1999) was adopted to capture the rate of STD of EPSCs and FF-IPSCs from experimental data (Figure 4-12). As a metric of input discriminability, the maximum potential achieved through summation of five EPSPs following stimulation with regular trains at between 1-200ms inter-stimulus interval (ISI, i.e. 1000-5Hz) was used (Figure 4-13). The strength of FFI at each stimulation frequency was again systematically increased between 0~10 to explore the potency of FFI. With no FFI, voltage summation was constrained as a function of input frequency, i.e. achieved when stimulating at frequencies that beat the decay kinetics of model EPSPs; the wild-type model showed summation at ISIs faster than 104ms (>10Hz), achieving 170% amplitude summation relative to the initial EPSP following 50Hz stimulation, and 200% following 100Hz. Broader EPSPs in model Fmr1-KO responses slightly promoted summation at lower frequencies (ISIs faster than 110ms, >9Hz) but more strikingly showed exaggerated
sensitivity to progressive increases in input frequency (180% summation at 50Hz, 232% at 100Hz). Introduction of moderate strength FFI (G/A ~1) to the wild-type model could efficiently prevent voltage summation at low frequencies. Increasing G/A ratios progressively raised the high-pass cut-off frequency until temporal coincidence detection could be achieved with sub-millisecond precision (i.e. no summation for IEI>1ms) as previously reported (Gabernet et al., 2005) with G/A>3.5, close to the mean wild-type experimentally derived value. In the Fmr1-KO model however, the efficacy of FFI in preventing summation during short trains of low frequency inputs was reduced, such that strong depolarisation was still possible with inhibition provided at moderate G/A ratios (e.g. Figure 4-14a, G/A=1). This behaviour compromised low-frequency input rejection and supported largely unconstrained excitation during high frequency stimulation, as demonstrated in Figure 4-13 and Figure 4-14. Sub-millisecond input coincidence detection required stronger (G/A>4.5) FFI to enforce relative to the wild-type model, however excessive summation relative to wild-type conditions was still observed under these conditions (Figure 4-14b), with inter-model divergence becoming progressively confined to higher stimulation frequencies. Strikingly, the amount of additional FFI strength required to achieve comparable summation performance to the wild-type model was predictive of experimentally derived mean G/A ratios. It is therefore proposed that the moderate shift towards stronger FFI strengths found in Fmr1-KO neurons in the present study could act as a beneficial, if inefficient, re-tuning of overall FFI performance when combined simultaneously with other circuit defects.
Together, these simulation results suggest that changes to \textit{Fmr1-KO} intrinsic, synaptic and presynaptic neuronal physiology, as well as seemingly small alterations to the precise timing and strength of the local FFI circuit, interact dynamically to profoundly change the frequency-dependent voltage dynamics of thalamorecipient L4 excitatory neurons.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure49}
\caption{Strategy for simulating effects of altered feed-forward inhibition on temporal window for integration at thalamocortical synapse}
\end{figure}

\textbf{Left:} Model glutamatergic and GABAAergic (“Exp2Syn”) synaptic inputs, fit to synaptic kinetics and short-term plasticity of genotype mean experimental data. For a fixed EPSC strength, effects of artificially varying IPSC strength were examined.

\textbf{Centre:} Model somatic currents were injected into a simulated passive RC soma with intrinsic properties matching genotype mean experimental data.

\textbf{Right:} Frequency dependence of voltage summation behaviour was examined as a function of steady state FFI strength. Simulated peak voltage deflection was used as a readout of coincidence detection performance. Other contributing parameters were examined by investigating the effect of individual \textit{Fmr1^{-Y}} synaptic phenotypes. The leaky integrating model was then expanded to provide spiking behaviour.
Figure 4-10 Simulated Fmr1-KO somatic voltage responses during feed-forward inhibition show altered kinetics across a range of physiologically-plausible GABA/AMPA ratios.

Example responses for the full Fmr1*+/Y and Fmr1*+/Y models showing the effect of FFI on the simulated voltage response following a single stimulus (below, black). Note the larger, slower EPSP in the absence of FFI (G/A=0), and the progressively larger IPSP for the Fmr1*+/Y model with increasing FFI strength.
Figure 4-11 Altered kinetics of model Fmr1-KO voltage responses during single stimulus feed-forward EPSC-IPSC sequences are contributed to by divergent physiological phenotypes

a). Simulated responses from Figure 4-10 shown collapsed and superimposed to highlight changes to EPSP shape both in the absence of, and with the addition of FFI for the Fmr1+/Y model.

b). Model-specific effects on the shape of the simulated voltage response (left: EPSP amplitude normalised to that of the Fmr1+/Y without FFI; right: EPSP duration at half-maximal amplitude), as a function of FFI strength (simulated G/A ratio). Insets expand responses for G/A=0–2. Also shown are the effects of replacing individual Fmr1+/Y parameter groups with those of Fmr1−/Y genotype means. Note the pronounced shoulder effect for FFI strength of G/A~2, above which little extra EPSP attenuation or truncation is observed.
4.5.2.3. Simulated FFI (3): Abnormal thalamocortical integration supports exaggerated coincidence detection in the *Fmr1-KO* model

In the previous simulation, regular trains of model inputs were used to explore the frequency sensitivity of synaptic integration in a single passive compartment model soma. While this stimulus paradigm is largely consistent with some previous *in vivo* studies that have explored the kinetics of thalamocortical inputs during forced whisker deflections (e.g. Brecht and Sakmann 2002; Higley and Contreras 2006) it is unlikely that such regular thalamocortical APs arise during naturalistic whisking or active touch. Rather, coincidence detection between multiple, temporally distributed thalamic afferents has been suggested to provide rich and nuanced high frequency information via spike correlations among multiple inputs (Petersen et al., 2008; Bruno and Sakmann, 2009). This coding scheme is thus more likely to provide sub-millisecond spike correlations that necessitate fast FFI as discussed above (Gabernet et al., 2005) and are hard to capture in bulk stimulation experiments in slices. A more biologically realistic scenario would therefore involve simulating coincidence detection between multiple thalamocortical input streams whilst varying inter-stream statistical correlations. However, since distributed thalamic inputs onto L4 excitatory neurons target multiple dendritic segments within ~100μm of the soma (White and Rock, 1979; Feldmeyer et al., 1999; Silver et al., 2003), substantial assumptions would be necessary in addition to current experimentally obtained parameters. Therefore, a simplifying situation is presented whereby varying degrees of jitter are introduced into the previous single-stream model such that each
inter-spike interval is convolved with a Poisson distribution to introduce higher degrees of temporal coincidence between input spikes. Again, both EPSC and FF-IPSC amplitudes have linked use-dependence fit to experimentally defined parameters. This scenario is likely more consistent with run-down of FFI than of excitatory inputs since multiple afferent TC streams will target individual projecting FS interneurons, whereas individual TC inputs will depress largely independently, despite the presence of multiple release sides per axon. Under these conditions of Poisson-jittered inputs (Figure 4-15), simulated voltage summation in response to 5 model stimuli closely followed that of regularly timed inputs (compare Figure 4-15c and Figure 4-14a), again demonstrating the role of FFI in sharpening the window for temporal integration at TC inputs. These results suggest that the predicted effects of abnormal FFI circuitry in Fmr1-KOs are not limited to the regular input patterns imposed in slice experiments in the present study. Furthermore, as a proxy for fast temporal correlations arising between synaptic inputs from multiple thalamocortical axons during awake whisker usage, these results show that high frequency inputs escape FFI to a greater extent in Fmr1-KOs to achieve stronger voltage summation, thus contributing to exaggerated high frequency sensitivity.
Figure 4-12 Fitting model parameters to short-term depression of excitatory and inhibitory synaptic inputs obtained from experimental data – example procedure for mean wild-type responses

a). Best-fit parameter estimation for STD of model EPSCs (black crosses and joining lines marking five regular stimulation times) converged onto mean experimental fits (blue, normalised to steady-state amplitude as in Figures 4-7,4-8). Note the trade-off between faithfully matched paired-pulse depression behaviour at all frequencies between 5-50Hz and convergence of depression to plateau values during ongoing stimulation. b). As a), but showing parameter estimation (black) converged to mean FF-IPSCs depression (red). c). Rate of FFI run-down predicted by best-fit model parameters (black, ratio between black fits in b+a) normalised to steady-state strength, and compared to mean ratios between direct fits to experimental data as in Figure 4-8 (shown green here).
Figure 4-13 Simulated voltage summation of 5 model synaptic inputs at regular frequencies: High-pass filtering of synaptic integration depends upon strength of feed-forward inhibition

a). For FFI strengths of G/A=0–4, simulated subthreshold voltage deflections for the full $Fmr1^{-/-}$ and $Fmr1^{+/+}$ models (red and blue, respectively) in response to five regular stimuli at 5-200ms Inter-stimulus interval (ISI), i.e. for 5-200Hz.

b). Maximum simulated voltage deflection for the two models plotted for the range 1<ISI<200, 0<G/A<5, highlighting high-pass frequency filter effect brought about with the addition of FFI. For the $Fmr1^{-/-}$ model, note the elevated voltage summation at large ISIs in the absence of FFI, and he reduced efficacy of FFI at restricting summation to fast ISIs.
Figure 4-14 Feed-forward inhibition is inefficient in Fmr1-KO simulations, permitting aberrant integration of low frequency synaptic inputs and excessive, unconstrained summation at higher frequencies

a). Simulation responses (5 stimuli) from Figure 4-13 replotted to highlight inefficient high-pass filtering provided by moderate strength FFI in the Fmr1+/Y model.

b). Differences in simulated frequency sensitivity of the two models expressed as their fractional difference in voltage summation performance (five stimuli) as normalised to the amplitude of their first stimulus response.
Figure 4-15 Simulated Poisson-jittered synaptic input trains times demonstrate exaggerated coincidence detection behaviour at the model *Fmr1-KO* thalamocortical synapse

a). Schematic representation of how jittered synaptic input trains were constructed using NEURON’s noisy stim-source routine. Black vertical lines are stimulus times, grey symbols schematise probability distributions of next stimulus times, with peaks regularly spaced at the given ISI. Parameters for noise between 0.1~10 were explored. b). Examples of simulated summation of 5 jittered stimuli, varying through 1-50ms modal ISI, with noise=0.8. Note the excessive voltage summation at short ISIs for the *Fmr1-/-* model with moderate strength FFI (G/A=2).

c). Frequency response profiles for the two models with varying strength FFI as in Figure 4-14a, but showing the effect of jittered stimulation (five trials overlaid for each strength of FFI).
4.5.2.4. Simulated FFI (4): Multiple parameter sensitivity analysis and simulated rescue of FFI performance in silico

Grouping of individual parameters was adopted in the previous simulations. Therefore, in addition to comparing full wild-type and Fmr1-KO model performances, it was possible to further dissect different combinations of wild-type and mutant parameters. This approach yields several benefits; namely, it provides deeper insight into the interaction between individual parameters under conditions of either normal or pathological function and identifies dominant phenotypes. Furthermore, such an approach probes sensitivity of the dynamic FFI system in the pathological Fmr1-KO state to different scenarios of simulated “rescue” of different aspects of abnormal physiology. For example, what would be the effect of reducing intrinsic excitability of recipient neurons with a pharmacological agent? Would it be necessary to additionally consider retuning the decay kinetics of FF-IPSCs to avoid potential over/under compensation?

Fmr1-KO FFI parameters were thus systematically replaced with their wild-type counterparts and frequency-dependent summation behaviour in response to 5 regularly timed inputs was explored for the full combinatoric set. This produced 16 possible scenarios, ranging from full Fmr1-KO to full wild-type, through 14 additional conditions (four parameter groups, two possible (mean) genotype values, i.e. $2^4 = 16$ possible scenarios: Figure 4-16a). As an index of disparity, the summed difference between genotypes across the $1<\text{IEI}<200\text{ms}, 0<\text{G}/\text{A}<10$ parameter space was considered (as in
Figure 4-14b). This metric was chosen since it spans both the range of FFI strengths found in Fmr1-KOs experimentally and also covers stimulation frequencies that are likely to arise during sensory exploration, albeit characteristic of whisking behaviour in adult mice.

The results of this analysis are shown in Figure 4-16b. Interestingly, while a general trend to better FFI performance was observed with more parameter groups “rescued” to wild-type values, it was possible to over- or under-compensate with specific parameter combinations and further degrade FFI performance. Overall, this supports the conclusion that a dynamic equilibrium between circuit-distributed defects exists in Fmr1-KOs such that careful consideration of a multi-faceted treatment strategy would be required to return circuit performance to “normal” ranges. In particular, it is conceivable that rebound circuit homeostasis could follow targeted treatment of individual physiological defects. With this in mind, when considering the strongest single phenotype candidate for rescue, individual repair of either postsynaptic intrinsic excitability or Ex-In delay phenotypes (conditions “1000” and “0010”, respectively in Figure 4-16b) could improve mean performance by over half compared to the full Fmr1-KO simulation36, whereas these two effects combined (condition “1010”) had a deleterious mean overcompensation effect even compared to the original Fmr1-KO situation.

36 Albeit reaching the same mean value through different mechanisms: reducing excitability to WT levels was broadly effective except during very high stimulation frequencies, whereas shortening Ex-In lags caused an overcompensation for high G/A cases during prolonged stimulation at low frequencies, with little effect at high frequency stimulation.
Figure 4-16 Circuit-distributed contributions of individual *Fmr1-KO* physiological parameters to abnormal voltage summation behaviour in thalamocortical integration model: simulated phenotype rescue *in silico*

a). Whole parameter space explored for 16 different possible combinations of simulated *Fmr1*<sup>+/Y</sup> and *Fmr1*<sup>-/Y</sup> conditions (4 parameter groups, two possible genotypes, i.e. 4<sup>2</sup> combinations). Voltage summation performance for five stimuli, normalised to the first EPSP amplitude and divided by the full *Fmr1*<sup>+/Y</sup> condition as in Figure 4-14b. Colour scale is adjusted such that white is no change relative to wild-type, hot and cold colours are excessive and weaker depolarisation, respectively.

b). For each parameter combination, mean summation performance across the whole 1<SI<200ms and 0<GA<10 range is shown compared to that of the wild-type simulation (i.e. full *Fmr1*<sup>-/Y</sup> model performance=0), as a function of the number of “repaired” parameter groups.
Impact on coincidence detection performance

Parameter Group Rescued
- Short-term plasticity  xxx1
- Ex-In delay          xx1x
- Synaptic kinetics    x1xx
- Intrinsic excitability 1xxx

Excess Summation (normalized to WT)

(a) Impact on coincidence detection performance

(b) Mean performance impact relative to WT

Graph showing the impact on coincidence detection performance with parameters grouped and color-coded for excess summation.
This scenario could in turn more closely approximate wild-type performance with the further rescue of short-term plasticity or synaptic kinetics (conditions “1011” and “1110”, respectively).

An analogous pharmacological strategy might aim to reduce Ex-In latency in Fmr1-KO mice by enhancing speed of TC integration in FS interneurons and subsequent AP transmission onto EX neurons. This could conceptually be achieved by administering a compound (e.g. a TASK channel modulator) to affect FS cell input resistance, and thus membrane time constant. Such a compound would therefore need a cell-type specific action to prevent simultaneous interaction with excitatory neuron excitability (to avoid condition “0101”). If this hypothetically specific compound were not available, a combined therapeutic strategy with the addition of a selective blocker of slow GABAergic currents, or positive allosteric modulator of extant fast GABA<sub>A</sub> subunits might be considered. A final strategy would be to identify a core circuit deficit unnameable to direct pharmacological manipulation and aim to indirectly affect it through targeted treatment of known homeostatic interactions.

4.5.2.5. **Simulated FFI (5): Altered coincidence detection performance in Fmr1-KO model predicts distorted spiking behaviour**

Although simplified sub-threshold voltage dynamics were explored in the previous model simulations, realistic synaptic currents and time constants, input resistances and conductance reversal potentials were employed. The evoked EPSP amplitudes and thus the predicted excessive voltage summation obtained can therefore be considered biologically plausible.
Correspondingly, these responses are likely capable of depolarising thalamorecipient neurons above spiking threshold and altering the recruitment of spiking in postsynaptic Fmr1-KO neurons.

This suggests that the relaxed temporal input selectivity of Fmr1-KO L4 neurons could enhance their recruitment to fire by low frequency inputs. This is tested in silico in Figure 4-17 and 18 during regular and Poisson-jittered stimulation, respectively, by adding a minimal set of active conductances to the previously used model soma to approximate “leaky integrate and fire” behaviour, maintaining the original subthreshold passive membrane parameters. To normalise for differences in input resistance between wild-type and Fmr1-KO models, stimulation strength (EPSC peak conductance) was adjusted for each genotype to provide a single-EPSP depolarisation of ~5mV (in the absence of FFI) from a simulated leak reversal potential (a proxy for resting potential) of -65mV. Coincidence detection and integration of multiple TC inputs was therefore necessary to depolarise by 10mV to reach AP firing threshold (-55mV).

With 5 regularly timed inputs (Figure 4-17), in the absence of FFI (G/A=0) the Fmr1-KO model could be recruited to fire an AP with stimulation at ISIs faster than 55ms, while the wild-type model required inputs faster than 30ms ISI. Addition of moderate strength FFI (G/A=2) narrowed the wild-type model coincidence detection window to restrict spiking in to ISIs shorter than 5ms, but the Fmr1-KO model would still fire at >15ms ISI. Correspondingly, strong FFI (G/A =4) was necessary to restrict Fmr1-KO -model coincidence detection to sub-5ms ISIs, at which strength the wild-type model required >5 stimuli to evoke a spiking response (data not show)
at ISIs slower than 1ms. Similarly, providing five Poisson-jittered stimuli with mean ISIs between 1-200ms (Figure 4-18) evoked AP firing with a frequency sensitivity that closely followed the subthreshold voltage responses of both genotypes (Figure 4-15). Taken together, these simulations display clear genotype-specific dependent changes to both subthreshold voltage summation and recruitment of spiking behaviour in response to behaviourally salient TC input frequencies. These results thus provide a strong prediction that altered performance of the Fmr1-KO FFI circuit should impact upon the frequency-dependent recruitment of cortical network activity by ascending thalamocortical inputs. It should be noted that the above simulations likely underestimate these effects, both through slight under-fitting of use-dependent FFI rundown, and the lack of voltage-gated NMDA conductances in the model. The latter is of particular significance, since an amplifying effect of NMDA has been proposed at TC inputs to L4 excitatory neurons, supporting the frequency-dependent nonlinear integration of sensory inputs in both visual and somatosensory cortices (Krukowski and Miller, 2001; Lavzin et al., 2012).

Figure 4-17 A spiking model of Fmr1-KO thalamocortical temporal integration demonstrates aberrant recruitment of action potential firing by low frequency model synaptic inputs as a result of inefficient feed-forward inhibition

a). Fmr1+/Y and Fmr1-/- model responses to 5 stimuli at ISIs between 1-100ms for FFI strengths of G/A=0, 2 and 4. Dotted lines indicate peak depolarisation for each simulation condition: ISIs that caused simulated AP firing show an obvious jump in maximum depolarisation. Insets show all simulations for that FFI condition overlaid (i.e. 1:100ms with 5ms resolution).

b). Binary description of the models’ temporal resolution by whether or not each simulation condition evoked an AP following summation of five EPSPs. To normalize stimulation strength to account for elevated input resistance in the Fmr1-/- model, excitatory peak conductances were adjusted such that the first EPSP caused the same voltage deflection for each genotype.
a  Coincidence detection performance

G/A = 4

G/A = 2

G/A = 0

Maximum depolarization (mV)

Inter-stimulus interval (ms)

b  Model temporal resolution

G/A ratio

Inter-stimulus interval (ms)

\( Fmr1^{+/Y} \) model

\( Fmr1^{-/Y} \) model

\( = \) Fired A.P.
Figure 4-18 Exaggerated coincidence detection of high frequency Poisson inputs in Fmr1-KO thalamocortical integration model supports excessive spiking due to inefficient feed-forward inhibition

Simulations and quantification in a) and b) are as in Figure 4-17, but use Poisson-jittered stimulus trains as in Figure 4-15. Binary quantification of temporal resolution in b) shows results for a single trial for each simulation configuration.
4.5.3. Current-clamp characterisation of dynamic thalamocortical FFI

4.5.3.1. Exaggerated thalamocortical EPSPs in Fmr1-KO neurons with weak to moderate strength FFI

It was necessary to validate the predictions made in the above simulations by examining ex vivo the input frequency dependence of both individual neuron membrane potential responses, and their contribution to the recruitment of cortical network activity by thalamocortical stimulation. The strength of TC-evoked FFI in individual voltage-clamped wild-type and Fmr1-KO Ex. neurons was first recorded as described previously before switching to current clamp recording configuration to examine the effect of FFI on the neurons’ voltage response (Figure 4-19a). Neurons were maintained at -65mV, close to mean resting membrane potential for both genotypes. No significant differences between either evoked thalamocortical EPSC amplitudes (Figure 4-19b,c) or stimulation strengths (data not shown) were observed between genotypes. Additionally, there were no changes to EPSP onset kinetics (rising speed or time - Figure 4-19d).

With single VB stimuli, wild-type Ex cells with stronger FFI showed a trend towards shorter TC EPSP half-widths (examples shown in Figure 4-20, plotted with reference to FFI strength in Figure 4-21a), as reported previously (Daw et al., 2007a; Chittajallu and Isaac, 2010), confirming that the duration of TC evoked responses was modulated by FFI.
Figure 4-19 Recording thalamocortical excitatory post-synaptic potentials (EPSPs) in current-clamped Layer 4 excitatory neurons

a). Left, schematic experimental configuration showing whole-cell recording from Layer 4 Ex neuron. Right, example traces showing TC-evoked responses and the relationship between voltage-clamped EPSC and EPSP recorded in current clamp whilst biasing the membrane potential to -65mV. b). Evoked EPSC amplitudes (or range of TC stimulation strengths used) were not significantly difference between genotypes. Data points are neurons, bars are mean±SEM (p=0.83, t-test, N (neurons); 17 Fmr1+/Y, 36 Fmr1−/Y) c). The relationship between the evoked EPSC and EPSP amplitudes was significantly steeper in Fmr1+/Y recordings. Data points are neurons; lines show best±95% CIs for linear fits through the origin (slope: Fmr1+/Y 0.028±0.004, Fmr1−/Y 0.035±0.002, *p=0.007, extra sum-of-squares F-test, N’s as in b). d). Onset kinetics for TC-evoked EPSPs (rise slope and time) were unaffected in Fmr1+/Y recordings (p>0.05, t-test, data are neurons from b).
Figure 4-20 Example thalamocortical EPSPs recorded from Layer 4 excitatory neurons, recorded in current clamp configuration at -65mV.

Note the progressive curtailment of EPSP duration with increasing FFI strength (scale 5mV,50ms).
Figure 4-21 The shapes of thalamocortical EPSPs, and effects of FFI are distorted in Fmr1-KO neurons.

Plotted points are neurons (N (neurons) = 19 Fmr1+/Y, 36 Fmr1−/Y), bars are mean±SEM, and asterisks denote significance (p<0.05). Population division at G/A=2 is marked with dashed lines on each figure.

a). Relationship between strength of FFI and EPSP half-widths (ms)

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b). Relationship between strength of FFI and EPSP peak depolarisation from -65mV (mV).

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<th>Fmr1−/Y (mean±SEM)</th>
<th>p value (t-test)</th>
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<tr>
<td>Neurons with 0&lt;G/A&lt;2</td>
<td>4.72±1.27</td>
<td>7.72±1.37</td>
<td>0.09</td>
</tr>
<tr>
<td>Neurons with G/A&gt;2</td>
<td>2.55±1.46</td>
<td>4.68±1.00</td>
<td>0.12</td>
</tr>
</tbody>
</table>

c). Relationship between strength of FFI and depolarising EPSP integral from holding -65mV (mV.ms).

<table>
<thead>
<tr>
<th>Data classification</th>
<th>Fmr1+/Y (mean±SEM)</th>
<th>Fmr1−/Y (mean±SEM)</th>
<th>p value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All neurons</td>
<td>160±97.5</td>
<td>323±66.3</td>
<td>0.17</td>
</tr>
<tr>
<td>Neurons with G/A=0</td>
<td>N/A</td>
<td>224±78.3</td>
<td>N/A</td>
</tr>
<tr>
<td>Neurons with 0&lt;G/A&lt;2</td>
<td>201±125</td>
<td>569±122</td>
<td>*0.02</td>
</tr>
<tr>
<td>Neurons with G/A&gt;2</td>
<td>24.0±12.0</td>
<td>219±99/4</td>
<td>0.21</td>
</tr>
</tbody>
</table>
Similar trends towards lower EPSP amplitude and smaller depolarising EPSP integral were observed with increasing G/A ratios. These experimental findings are broadly consistent with model predictions made in Figure 4-11b, where potent constraining effects on EPSP shape were obtained by moderate strength FFI (G/A<2), with minimal additional influence observed by further increasing G/A ratios.

Fmr1-KO neurons however, when considered as a group regardless of FFI strength, showed a significant shift towards broader EPSPs compared to wild-type neurons. This effect was largely dominated by a striking increase in EPSP duration in a sub-population of cells receiving weaker FFI (G/A<2). Additionally, EPSP half-widths from these cells were indistinguishable from Fmr1-KO neurons lacking FFI, suggesting that this strength range was largely ineffective in curtailing the temporal voltage kinetics. A similar effect was observed using another measure, the EPSP depolarising integral (Figure 4-21c). For Fmr1-KO neurons with stronger FFI (G/A>2), TC-evoked EPSPs were on average slightly broader and higher amplitude than their wild-type counterparts. In partial agreement with the above modelling predictions therefore, these data suggest that moderate strength FFI (G/A<2) has reduced potency at attenuating EPSP amplitudes in Fmr1-KO neurons. However, the longer TC-evoked EPSP durations go against the model predictions in Figure 4-11 for all but weakest FFI strengths. Notably, even in the presence of strong FFI, broad EPSPs were seen, suggesting that FFI in Fmr1-KOs is rendered inefficient at constraining EPSP duration. It is possible that independent cell-to-cell variability of individual FFI parameters, coupled with the activation of
NMDA receptors (not accounted for by model responses) account for the majority of this effects on EPSP duration, and may explain disparity with simulated responses.

4.5.3.2. Abnormal coincidence detection and summation of paired-pulse thalamocortical stimuli in *Fmr1-KO* Layer 4 excitatory neurons

To investigate how abnormal short-term plasticity of TC EPSCs and FF-IPSCs in *Fmr1-KOs* modulating voltage summation and affect the coincidence detection of TC inputs, coincidence detection of paired stimulation at high frequencies was first considered. **Figure 4-22** presents example current-clamp recordings from Layer 4 Ex cells in which TC inputs were stimulated at ISIs between 5-50Hz in 5ms increments. To highlight the effect of FFI compared to the “innate” EPSP (i.e. minimising the effect of FFI), each experiment was repeated twice, holding cells both at -60mV (with approximately 10mV of Cl- driving force), and at -70mV (effectively no Cl- driving force). EPSP amplitudes are shown normalized to the first evoked EPSP to compare effects on summation.
**Figure 4-22** Example summation of paired-pulse thalamocortical inputs in Layer 4 Excitatory neurons with weak and strong feed-forward inhibition

**a-b).** Example recordings showing voltage summation of two TC stimuli for ISIs between 5-50ms, showing average of ~5 trials (mode), presented in pseudo-random order. For each trial, traces are scaled to normalise the amplitude of the first EPSP to 1. Each stimulation sequence was repeated while holding the cell at -60mV and -70mV($E_{\text{GABA}_a}$ shown in grey). Note the role of FFI in constraining EPSP summation, but strong summation achieved in the $Fmr1^{-/-}$ example despite the presence of strong FFI.

**c).** Effect of biasing neurons from -60mV to -70mV on their TC integration performance in a small subset of recordings. Left: peak summation (relative to 1st EPSP amplitude). Centre: ISI that caused the greatest summation. Right: Classification of summation response at each holding potential by best ISI (5-15ms: “high-pass”, 20-35ms: band-pass”, 40:50ms: low-pass).
**a** Low FFI strength examples

- $G/A=0.2$
- $G/A=2.4$

**b** High FFI strength examples

- $G/A=0.6$
- $G/A=6$

**Holding potential**

- $-70mV$
- $-60mV$

- **Fmr1**
- **Fmr1**

- **Fmr1**
- **Fmr1**

**Best ISI (ms)**

- **Fmr1**
- **Fmr1**

**Max summation (x 1st EPSP)**

- **Fmr1**
- **Fmr1**

**Filter classification**

- **High Pass**
- **Band Pass**
- **Low Pass**

$n=6$
In the wild-type example neuron with only weak FFI (Figure 4-22a, left), changing holding potential had little effect on the relative frequency sensitivity of voltage summation, such that similar degrees of summation were achieved across the full range of inter-pulse delays at both -60mV and -70mV. For the Fmr1-KO neuron with weak FFI (Figure 4-22a, right), robust summation could be achieved across a similarly broad range of ISIs to the wild-type cell, largely independent of holding potential changes imposed. However a pronounced increase in summation was observed when inputs were stimulated at 10-15ms ISI when the cell was held at either -60mV or -70mV. Here, pronounced summation and putative voltage-gate conductance activation occurred, often eliciting an AP after only two stimuli. EPSP decay kinetics on the second EPSP were also noticeably slower under all conditions in the Fmr1-KO neuron compared to the wild-type. In the example wild-type cell with strong FFI (i.e. G/A>2, Figure 4-22b left) however, pronounced EPSP-IPSP sequences were recorded at -60mV on both the first and second response, albeit with broader EPSPs on the second, consistent with moderate depression of FFI. Here, EPSP kinetics were shorter such that summation was putatively restricted to ISIs <5ms (i.e. faster than tested) and FFI efficiently prevented summation at all ISIs tested. This effect could be abolished by repeating stimulation at -70mV where correspondingly, above-unity summation was observed at all ISIs between 5~50ms. This highlights the role of FFI in controlling the voltage kinetics and improving coincidence detection performance of thalamocortical EPSPs. Conversely, for the Fmr1-KO example neuron with strong FFI, FFI was abnormal at -60mV such that EPSPs at short ISIs were
prevented from summing, but depression of FFI during longer ISIs led to robust summation that was capable of firing APs after just two stimuli. AP firing was blocked by hyperpolarising the cell to -70mV (polysynaptic input was not observed), suggesting that firing was driven by thalamocortical coincidence detection. Furthermore, above-unity summation could be achieved at -70mV at all ISIs tested in this example cell. Interestingly, this suggests that despite receiving strong FFI, altered coincidence detection in this Fmr1-KO neuron emerges as a function of abnormal FFI such that input frequency sensitivity transforms from “all-pass” to “low-pass/band-stop” filtering rather than to “high-pass” upon depolarisation (and introduction of FFI) as in the wild-type example. This hypothesis is explored further in a small number of neurons in Figure 4-22c. As expected, in the majority of neurons of both genotypes depolarising cells to -60mV (thereby introducing FFI) reduced summation relative to at -70mV. No consensus shift in the ISI promoting peak summation was observed (i.e. the “best ISI”) for either genotype when holding potential was changed, suggesting that the sampled neurons had a diverse range of ISI preferences for maximal summation that was at least contributed to by summation properties in the absence of FFI. Overall, it was possible to promote band-pass towards high-pass filtering in 2/4 wild-type neurons tested upon depolarisation from -70mV to -60mV. A similar effect was observed in some Fmr1-KO neurons, however, 2/8 tested cells showed strong low-pass effects that were insensitive to depolarisation since they lacked FFI (G/A =0 for both of these neurons).
Can these effects be explained by model predictions? Figure 4-23a shows simulated paired-pulse summation across the same range of ISIs (5-50ms) for genotype-mean parameters as before. Similar to results with five model stimuli, (c.f. Figure 4-13), simulations lacking FFI showed excessive paired-pulse summation across a broad frequency range. Graded introduction of FFI led to a progressive increase in high-pass filter cut-off frequency for both genotype models. However, stimulation at frequencies above this led to excess summation in Fmr1-KO simulations.

**Figure 4-23** Simulated thalamocortical paired-pulse summation response for wild-type and Fmr1-KO models

a). Simulated voltage responses for two stimuli separated by ISIs of 5-50ms in 5ms increments (all ten simulations shown overlaid).

b). Paired-pulse summation performance explored for the parameter space 0<G/A<10, 1<ISI<50ms. Left and Centre: Summation is shown normalised to the first EPSP amplitude for each simulation as in Figure 4-14. Right: Fmr1+/Y summation response is divided by the Fmr1−/Y response to compare the two models’ performance.
This effect is summarised in Figure 4-24b. Greatest disparity between model performances was observed for stimulation at ISIs <10ms and G/A ratios 4-6. Correspondingly, it was not possible to recreate low-pass paired-pulse summation responses observed in some Fmr1-KO neurons using genotype mean parameters. However simultaneously increasing the rate of FFI short-term depression while lengthening IPSC decay kinetics could approximate this behaviour (data not shown), suggesting that low-pass effects arise in Fmr1-KO neurons that have this particular combination of co-varying phenotypes.

Experimentally derived thalamocortical voltage summation behaviour at -60mV is further examined as a function of steady-state FFI strength in Figure 4-24. Figure 4-24a replots example neurons from Figure 4-21a showing paired-pulse summation between 5-50ms ISI. As before, neurons were grouped as receiving either weak (G/A<2) or strong (G/A<2) FFI (quantified in Figure 4-24b). For wild-type neurons, strong dependence was observed for summation behaviour on steady-state G/A ratio and, indirectly, EPSP duration. Neurons with weak FFI showed slight high-pass filter characteristics of voltage summation during paired-pulse stimulation. These cells typically displayed above-unity summation at ISIs as slow as 15ms on average, with relatively broad and variable EPSP durations on the second stimulus. Conversely, for wild-type neurons with strong FFI, EPSP durations were considerably less variable on the second stimulus, with half-widths typically around 10ms. The frequency-dependence of summation was variable between neurons but largely remained at unity for all ISIs tested, suggesting that sub-5ms stimulation
was necessary to evoke voltage summation. For *Fmr1-KO* neurons with no or weak FFI however, little evidence of a high-pass filter effect during paired-pulse stimulation were observed. Successful voltage summation could be evoked at all ISIs tested and was significantly elevated above wild-type responses for ISIs <30ms. Similarly, EPSP duration on the second stimulus was both broader and highly variable between neurons and was significantly broader than wild-type values at ISIs <15ms. The opposite was observed for *Fmr1-KO* neurons receiving strong FFI. Second EPSP half-widths were very short and were typically riding on top of a large IPSP. Consequently, summation at short ISIs (typically 10-25ms) was markedly sub-unity in the majority of cells, returning to unity at longer ISIs.

**Figure 4-24** Altered summation of thalamocortical inputs in Layer 4 excitatory *Fmr1-KO* neurons

*a*. Shows **Figure 4-21a** replotted to show the effect of FFI strength and single EPSP half-width on paired-pulse summation behaviour. Example neurons show paired-pulse responses as in **Figure 4-22a**, normalised to the amplitude of the first EPSP from a holding potential of -60mV.

*b*. Paired-pulse summation behaviour of cells with weak and strong FFI (with G/A ratios below and above 2, respectively) quantified separately. Duration at half-height of the second EPSP (top) and maximum summation (bottom, normalised to the first EPSP amplitude) are shown. Asterisks indicate individual ISIs that yielded significantly different results between genotypes (p<0.05, t-test, $N$ (neurons) = Weak FFI, 6 *Fmr1+Y*, 5 *Fmr1-Y*; Strong FFI, 5 *Fmr1+Y*, 6 *Fmr1-Y*).
Neurons with Weak FFI (G/A<2)  Neurons with Strong FFI (G/A>2)

![Graph showing the relationship between ISI (ms) and Fold x 1st EPSP half-width (ms) for neurons with different FFI strengths.](image)

**b**

- **ISI (ms)** vs. **Fold x 1st EPSP**
  - Neurons with Weak FFI (G/A<2)
  - Neurons with Strong FFI (G/A>2)

- **Half-width**
- **Summation**

* indicates statistical significance.
These paired-pulse data demonstrate that compared to wild-type recordings, a stronger divergence between the voltage summation behaviour of neurons receiving strong and weak FFI exists in *Fmr1-KOs*. This suggests that during the high-frequency firing of thalamocortical neurons associated with whisker touch onset (Simons and Carvell 1989; Petersen et al. 2008), L4 neurons in *Fmr1-KOs* will undergo different input integration responses depending on the strength of the FFI they receive. From this data, it is conceivable that during the first two touch-related thalamocortical responses, cells with strong FFI will be less recruited by high frequency inputs than their wild-type counterparts, but will preferentially respond to thalamocortical inputs that arise at lower frequencies37. On the other hand, cells receiving weak/no FFI are sensitive to a wider range of input frequencies, in particular inputs that are arise at higher frequencies. These cells are perhaps more likely to fire in response to early contact as they will integrate a broader range of input frequencies – losing the ability to discriminate between them.

During ongoing trains of thalamocortical inputs however, since feed-forward inhibition runs down faster at *Fmr1-KO* thalamocortical inputs compared to wild-types in the present dataset (as presented in Figure 4-6,7,8), G/A ratios depress rapidly, dynamically changing the coincidence detection characteristics of neurons. Thus, Layer 4 cells receiving initially strong FFI will lose the high-pass response specialisation enforced by FFI and the population of Layer 4 excitatory cells will become biased towards low-pass input transformation (i.e. capable of integrating more broadly

37 Although discussed as “low-pass” filtering behaviour above, since <5ms ISIs were not tested, it is conceivable that a closer filter description may be “band-cut”.

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timed input). Overall this means that when stimulated with short trains of TC inputs, rather than isolated paired simulation, FFI will be ineffective at preventing summation and therefore ongoing stimulation at frequencies that overtake the EPSP decay kinetics in the absence of FFI will be capable of evoking unconstrained summation. Finally, Fmr1-KO neurons have longer membrane time constants than their wild-type counterparts (Figure 3-4) and Fmr1-KO neurons receiving weak FFI have by default larger and longer thalamocortical EPSPs for a given EPSC amplitude (Figure 4-20,1). It is therefore likely that EPSP broadening as a result of FFI run-down will permit summation at lower stimulation frequencies in Fmr1-KOs, with excessive summation observed at higher frequencies compared to wild-types. This section has developed understanding of how the voltage summation of wild-type and Fmr1-KO neurons is differentially affected by brief pairs of stimuli associated with early contact. How does the loss of FMRP affect cortical responses to repetitive TC input characteristic of active tactile exploration? The simulations shown in Figure 4-13,14, suggest large frequency dependent changes - these predictions are tested directly in the next section.

4.5.3.3. **Broader coincidence detection at Fmr1-KO TC inputs to L4 excitatory neurons**

Sub-threshold voltage summation of TC-evoked responses was explored during bursts of repetitive stimulation at physiological frequencies between 5~50Hz, mimicking VPm firing patterns characteristic during exploratory whisking behaviour.
Graded frequency-dependent EPSP summation was observed in wild-type excitatory neurons, consistent with gradual FFI rundown during ongoing TC stimulation. Furthermore, wild-type cells responded with a modest frequency-dependence, with minimal voltage summation observed during stimulation at frequencies below 20Hz (mean summation as normalised to first EPSP amplitude: 120% after 5x5Hz and 167% after 5x10Hz patterns, Figure 4-25), such that the probability of reaching the overall peak depolarisation was unaltered by successive stimuli (Figure 4-26a).

**Figure 4-25** Excessive sensitivity to high frequency thalamocortical stimuli in Layer 4 excitatory Fmr1-KO neurons

**Top:** Example traces showing current clamp recordings (neurons held at -60mV) from representative Fmr1+/Y and Fmr1-/- Layer 4 neurons during repetitive thalamocortical stimulation at frequencies between 5-50Hz (i.e. ISIs between 200-20ms), averaged over 10 repeated trials and median filtered to reduce contamination from spiking. Voltage amplitudes are shown normalised to the amplitude of the first EPSP. Note the significant increase both in voltage summation and EPSPs broadening after the first stimulus in the train in the Fmr1+/Y example.

**Bottom:** Normalised peak depolarisation for each successive EPSP responses in a train of five TC stimuli. Asterisks indicate statistically significant differences between genotypes at individual stimulus responses (p<0.05, t-test, N= 7 (animals) for each genotype).
Figure 4-26 Altered kinetics of thalamocortical summation in Fmr1-KO neurons (data are from Figure 4-25)

a). For each stimulus in a train of five at frequencies between 5-50Hz, mean probability of achieving the overall maximum voltage for that neuron’s response train.

b). Use-dependent broadening of TC-evoked EPSPs was exaggerated in Fmr1<sup>−/−</sup> neurons during 5Hz stimulation (measured from the same recordings as in Figure 4-25; N’s and statistics are the same). Similar observations were made at higher stimulation frequencies, this data is not quantified due to potential experimental error in calculating true TC EPSP half-width in Fmr1<sup>−/−</sup> recordings, in which contamination by polysynaptic inputs from local network activity were frequency encountered.
At higher stimulation frequencies, modest summation occurred and remained graded, with maximum summed EPSP amplitudes doubling those of the first EPSP’s amplitudes only after 5x50Hz stimulation. Use-dependent broadening of EPSPs also occurred in a graded fashion (Figure 4-26b), again suggesting that FFI depression took place gradually, rather than in a step-wise fashion.

When compared to wild-type littermates, Fmr1-KO excitatory neurons showed two clear differences in their voltage summation behaviour. Firstly, progressive graded summation during low frequency burst stimulation (5x5Hz and 5x10Hz stimulus trains) was disrupted, such that the depolarisation maxima occurred with the highest probability after the second stimulus (Figure 4-26a). This finding supports data showing excessively rapid shutoff of FFI after only one or two TC stimuli at 5Hz, but progressive and excessive depression of subsequent TC-EPSCs (Figure 4-6,7,8). Furthermore, supported by simulated summation responses Figures 4-13 and 4-14, an effectively immediate and significantly excessive broadening of EPSP duration was observed after the first stimulus at 5Hz (Figure 4-26b).

Secondly, significantly exaggerated, unconstrained summation was achieved during TC stimulation at higher frequencies: mean depolarisation exceeded double the amplitude of the first EPSP after only three stimuli at 20Hz, with three-fold summation after 5x20Hz and nearly five-fold gain after 5x50Hz.
Together these data demonstrate a dramatic departure from the fidelity of normal TCs input frequency discrimination performed by Fmr1-KO Layer 4 neurons, contributed to in a predictable manner by multiple combinatoric phenotypic interactions. Does such an effect have a functional impact? Compromising the frequency filtering ability of individual SCs could cause aberrant TC-evoked spiking and subsequent recruitment of the excitatory circuit of L4, impairing its ability to reject incoming synaptic activity at low frequencies. Such a reduction in input discrimination could impair performance at parsing salient and non-salient sensory context.

4.5.3.4. Impaired frequency-dependent gating of Layer 4 network activity by TC input transformation in Fmr1-KOs

An attractive hypothesis emerging from the present data at this point is that the abnormal frequency sensitivity of Layer 4 cells to thalamocortical inputs contributes to a hypersensitivity to high frequency inputs during sensory processing. Such a change in frequency-dependent circuit gain could provide a direct mechanistic explanation for lower perceptual thresholds for high frequency vibrotactile sensation previously reported for patients on the Autism spectrum (Blakemore et al., 2006; Marco et al., 2011). To address this, the efficacy of different frequencies of TC burst stimulation at recruiting a sustained polysynaptic L4 network response was examined. This approach takes advantage of the highly recurrent architecture (Petersen and Sakmann, 2000; Lefort et al., 2009) of the L4 circuit to use a single whole-cell recording as a reporter of network activity.
In wild-type slices, burst stimulation of VB at 50Hz evoked (non-stimulus locked) polysynaptic network activity in L4 approaching 90% success on average between multiple trials (Figure 4-27). Responses were stereotypically all-or-none and similar time courses could be resolved by recording in current-clamp (Figure 4-27a) and voltage-clamp (data not shown) configuration from excitatory neurons. 20Hz stimulation at the same intensity yielded ~29% success rate. Thalamic stimulation frequencies below 20Hz were uniformly unsuccessful in evoking polysynaptic firing in wild-type slices. This frequency-dependence profile (summarised in Figure 4-27b) is in good agreement with that reported by previous studies performed on older tissue (>P18) for this paradigm (Beierlein et al., 2002; MacLean et al., 2005), suggesting that this circuit property is mechanistically mature by P10-11 and may be representative of older TC-evoked circuit behaviour.

Figure 4-27 Ectopic recruitment of Layer 4 recurrent network activity with low-frequency thalamocortical stimulation in Fmr1-KOs

a). Example current-clamp recordings from Layer 4 Ex. neurons (10 trials overlaid) showing transiently sustained network activity evoked by 5 repetitive thalamocortical stimuli at 5, 10, 20 and 50Hz. (scale bar: 100ms, 10mV). Note relaxed requirement of high-frequency stimulation in generating sustained intracortical activity in Fmr1<sup>+/Y</sup> slices.

b). Fraction of trials evoking network activity as a function of stimulus pattern. Stimulation frequencies below 20Hz could not evoke firing (p(AP firing)=0±0) in Fmr1<sup>+/Y</sup> slices, but with low-moderate probability in Fmr1<sup>−/Y</sup> slices. Asterisks denote stimulation frequencies demonstrating significantly elevated firing probabilities in Fmr1<sup>+/Y</sup> slices versus Fmr1<sup>−/Y</sup> slices (P<0.05, t-test, N= Fmr1<sup>+/Y</sup>: 12 slices from 8 animals; Fmr1<sup>−/Y</sup>: n=10 slices from 10 animals). The 5x50Hz stimulation pattern evoked firing with high reliability (p(spiking): Fmr1<sup>+/Y</sup> = 0.87±0.11, Fmr1<sup>−/Y</sup> = 0.85±0.09) for both genotypes.
Figure a: Comparison of Fmr1+/Y and Fmr1−/Y stimulation patterns at different frequencies (5Hz, 10Hz, 20Hz, 50Hz).

Figure b: Graph showing the fraction of trials recruiting spiking across different stimulation patterns (5x5Hz, 5x10Hz, 5x20Hz, 5x50Hz) with error bars.
In Fmr1-KO slices, L4 network activity could be ectopically recruited by significantly lower frequency thalamic stimulation (Figure 4-27b): ~17% and ~51% of trials at 5Hz and 10Hz respectively evoked responses showing AP firing and polysynaptic membrane potential activity, whilst the mean difference in the probability of evoking polysynaptic activity with 20Hz compared to 50Hz stimulation was markedly diminished from ~57% in wild-type slices to ~18% in Fmr1-KO slices. TC-evoked EPSC amplitudes were not significantly different between genotypes (WT: 112±20pA (n=12 slices from 8 animals); KO: 93±25pA (n=10 slices from 10 animals), p=0.67, t-test). This result confirms that the changes to the frequency-dependent amplification of TC inputs by Fmr1-KO excitatory neurons demonstrated in this chapter do indeed translate to an altered frequency-dependent gating of intracortical circuit activity in L4. Furthermore, similar to recordings from Ex neurons, network feedback was also visible in recordings from Layer 4 FS interneurons of both genotypes, showing highly correlated all-or-none successes and failures in paired recordings (Figure 4-28a). Thus, the probability of aberrantly evoking putatively network driven polysynaptic activity in Layer 4 FS interneurons with low frequency thalamocortical stimulation was significantly higher in Fmr1-KO compared to wild-type slices.

Figure 4-28 Fmr1-KO Fast-spiking interneurons receive circuit feedback during network activity ectopically recruited by low-frequency thalamocortical stimulation. a). An Layer 4 FS interneuron – Ex. neuron paired recording from an Fmr1+/Y slice. Ten trials showing consistent recruitment of network activity visible in the voltage and spiking response of both neurons. b). Quantification as in Figure 4-27, showing that increased sensitivity of Fmr1+/Y Layer 4 network activity to low frequency TC stimulation results in non-stimulus-locked polysynaptic activity (and occasionally AP firing) in FS interneurons. Asterisk indicates stimulation patterns that yielded statistically significant firing probabilities between genotypes (P<0.05, t-test, N= 5 animals of each genotype; 1 paired recoding per animal).
Fraction of trials recruiting network feedback in FS cells

![Graph showing the fraction of trials recruiting network feedback in FS cells. The graph compares Fmr1^+/Y and Fmr1^-/- genotypes across different stimulation frequencies. The x-axis represents stimulation frequency (Hz) from 0 to 60, and the y-axis represents the probability of network feedback (p(Network feedback)). The data points are marked with error bars, and a significant difference is indicated by an asterisk (*) between Fmr1^+/Y and Fmr1^-/- genotypes.](image)
4.5.4. Circuit behaviour with intact internal chloride concentration

Finally, Figure 4-29 provides a demonstration that the response of Layer 4 to thalamocortical stimulation behaves as documented by whole-cell recordings in this chapter is faithful to the response obtained when internal chloride concentration is unaffected by whole-cell perfusion.

Here, a connected FS-Ex. paired recording was obtained in a slice from an Fmr1-KO animal by using dual perforated patch recordings. Figure 4-29a shows the effect of thalamocortical FFI on the voltage response of the Ex neuron as recorded in current clamp configuration under three recording conditions. Firstly, when the cell was maintained hyperpolarised at its $E_{Cl}$ (blue), a large slow EPSP is observed, even though the FS cell is firing (not shown), demonstrating the slow kinetics of the cell’s innate voltage response in the absence of FFI. Secondly, by depolarising the Ex. neuron to its resting potential (-60mV), and again allowing the FS cell to fire during TC stimulation (red), strong FFI was observed, effectively truncating the PSP half-width and attenuating its amplitude. Finally, by repeating this stimulation while preventing the FS cell from firing by hyperpolarising it to a holding potential of -90mV, the effect of FFI was reduced, but not entirely abolished (green). Here, the remaining FF-IPSP was still visible, attenuating the EPSP amplitude, but the decay of the total voltage response was noticeably broader. This suggests that in this example Fmr1-KO neuron, at least two interneurons with projections left intact during slicing were providing FFI onto the postsynaptic Ex cell. Figure 4-29b shows a single

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38 Previously experimentally determined by evoking a unitary IPSC by firing the FS cell and changing the postsynaptic holding potential to minimise the response
trial response of the paired recording during TC stimulation at 10Hz. A strong reduction in FFI was observed in the Ex. neuron’s voltage response after the first stimulus, manifesting as a pronounced EPSP broadening and voltage summation on subsequent stimuli. Meanwhile, the connected FS neuron (green trace) fired APs in response to the first and second stimuli, but with subthreshold EPSPs only for the remainder of stimuli. Stimulation of TC inputs at five times at 20Hz was sufficient to overcome FFI and evoke transient intracortical network activity, as revealed by a sustained depolarisation and AP firing (Figure 4-29c). Both neurons participated in this activity, with 10/10 trials yielding a spiking response in the Ex neuron during a 200ms period after the last stimulus with a time-course that correlated well with that of (putatively) network-driven membrane fluctuations in the FS interneuron.

Together these example recordings suggest that whole-cell responses obtained in this chapter using dialysing internal solution are representative of the single-cell and network behaviour of neurons with intact internal chloride ion concentrations.
Abnormal FFI performance and aberrant low frequency recruitment of cortical network activity in an example $\text{Fmr1}\text{-KO}$ FS/Ex paired recording with intact internal chloride concentration

**a).** Effect of preventing the firing of a single FS interneuron on the FFI received by a postsynaptic Ex. neuron.  
**b).** Dynamics of FFI and TC responses during VB stimulation at 10Hz.  
**c).** Single trial (left) and 10 trials overlaid (right) showing recruitment of network activity with 100% success rate in both cells by $5\times20$Hz VB stimulation.

*Figure 4-29* Abnormal FFI performance and aberrant low frequency recruitment of cortical network activity in an example $\text{Fmr1}\text{-KO}$ FS/Ex paired recording with intact internal chloride concentration.
4.6. Discussion

4.6.1. Disruptions to FFI performance in Fmr1-KOs emerge from circuit-distributed changes

Sections 4.3.2 and 4.3.2.4 (Figure 4-11 and Figure 4-16) explored the dependence of thalamocortical EPSP shape and input frequency sensitivity behaviour (respectively) on co-variation between parameters such as postsynaptic excitability and timing of FFI. Different combinations of these parameters were found to produce a wide gamut of responses to conserved patterns of model inputs.

The data presented in this chapter and these simulations in particular offer insight into the potential diversity of filter functions performed by thalamorecipient neurons in Layer 4, and how this might be altered in Fmr1-KOs. As discussed above, biological variation in ion channel expression and other physiological parameters is thought to offer beneficial contributions to information processing in cortical networks.

These findings raise interesting questions regarding the efficacy of FFI at the Layer 4 circuit level in Fmr1-KO barrel cortex. Importantly, no correlation was observed between the input resistance of recorded neurons and the strength of FFI that they received for neurons of either genotype (wild-type: $R^2 = 0.09$, $p=0.37$, $N=17$ neurons; Fmr1-KO: $R^2 = 0.096$, $p=0.21$, $N=16$ neurons) suggesting that the strength of FFI develops indiscriminate of variability in postsynaptic excitability. Therefore, as a consequence of the independent distributions demonstrated in the present study, it is conceivable that the circuit possesses diverse combinations of FFI strength.
and intrinsic excitability. As explored below, one consequence of this could be to provide a continuum of synaptic filters, with some cells capable of being more easily recruited by TC inputs at a given frequency, and others requiring integration of more responses to fire. Neurons possessing combinations of high intrinsic excitability and no FFI were seen in Fmr1-KO recordings. The circuit level ramification of this combination might be that these neurons possess dramatically altered tuning to TC inputs and could be hypersensitive to sensory stimulation. Additionally, due to the increased mean input resistance in Fmr1-KO Ex neurons, stronger FFI is also predicted to be less efficient in these neurons. These hypotheses were tested through modelling in this chapter. Overall, it is likely that these changes distort population coding during sensory processing; a combination of weak FFI and strong postsynaptic excitability in a minority of cells in the barrel are predicted to be recruited to fire more easily in response to TC input, and thus contribute to “kindling” recurrent circuit activity in L4 in response to patterns of stimulation which would not normally be successful.

4.6.2. Comparison of the timing of altered FFI development and abnormal critical periods in Fmr1-KOs

In light of the delayed critical period maturation at Fmr1-KO TC synapses (Harlow et al., 2010), overtly reminiscent of delayed visual cortex critical periods following dark rearing (Kirkwood et al., 1995), it is pertinent to speculate to what extent disruption to the strength of FFI in Fmr1-KOs is
similar to the sensory deprived animal. This will be covered in the proceeding sections.

4.6.2.1. Role of GABAergic maturation in early critical period development

A role for precise maturation of the inhibition/excitation balance has been proposed in both opening (Hensch, 1998, 2004) and “gating” the closure (Kirkwood and Bear, 1994; Rozas et al., 2001) of critical periods in the visual cortex. Thus GABAergic signalling is recognised as playing a central role in coordinating the timing of activity-dependent visual circuit development. However, comparison to barrel cortex is difficult since the thalamocortical critical period occurs much earlier in barrel cortex, when GABA is still depolarising in many neurons in L4 and functional synaptic connectivity from FS interneurons is underdeveloped (Figure 4-1, and Daw et al. 2007), suggesting that GABAergic circuitry is still immature (Owens and Kriegstein, 2002). In the present study, maturation of [Cl\(_{\text{internal}}\)] did not appear different between wild-type and Fmr1-KOs neurons, at least between P8-11. However, recordings from L4 Ex neurons at P6-7 showed that while nearly half (41%) of wild-type neurons received detectable TC FFI, a significantly smaller fraction (13%) of Fmr1-KO neurons received FFI, and that G/A ratios on cells with FFI were significantly weaker in Fmr1-KOs (neurons with G/A ratio>0: 13/32 from 5 animals (WT), 20/23 from 7 animals (KO); mean±SEM G/A ratios excluding failures = 1.22±0.33 (WT), 0.28±0.09 (KO). *p=0.04 excluding, or **p=0.01 including cells with G/A=0, Mann-Whitney). Thus, TC FFI developmental maturation does appear to start later in Fmr1-KO barrel cortex compared to wild-types. Despite
defective GABA synthetic machinery reported in the Fmr1-KO cortex (Adusei et al., 2010), it is unlikely that this phenotype is the sole determinant (i.e. critical period “opener”) of the abnormally late developmental onset of thalamocortical plasticity in L4 shown by Harlow et al. (2010). In the referenced study, the authors showed near maximal TC potentiation at P5, a time at which the presence of FFI Fmr1-KOs is likely to be minimal, as predicted from data in the current study. The role of GABA in opening the critical period (as proposed by Hensch and colleagues in the visual system - referenced above) is further unlikely for the wild-type barrel cortex, where maximal potentiation of TC inputs is observed at P3 (Crair and Malenka, 1995), at which time FS and Ex neurons are unconnected in L4, and Ex cells receive no FFI (Daw et al., 2007a). Nevertheless, an insightful experiment would be to chronically administer benzodiazepines (or otherwise potentiate GABA$_A$, e.g. Hensch et al. 1998$^{39}$) during the first postnatal week in Fmr1-KOs. If critical period defects in TC LTP could be rescued in these mice, it would causally implicate defective GABAergic transmission in this phenotype and add weight to the hypothesis that GABAergic function impacts upon critical period timing in barrel cortex as shown previously for visual cortex$^{40}$. However, whether or not this would directly repair the developmental delay in maturation of FFI is uncertain. If developmental maturation of FFI is indeed a defining marker of the end of the critical period as suggested for the visual cortex, then data presented in this section is hard to interpret.

$^{39}$ This study elegantly demonstrated GABAergic control of visual cortex critical period timing by either reducing (by genetic knock-down of GAD65), or enhancing (with benzodiazepine administration) GABA transmission.

$^{40}$ Further evidence suggests that the mechanisms of TC LTP are distinct between visual (Wang et al., 2013) and somatosensory cortex (M. Daw, Personal communication).
In *Fmr1-KOs*, a split population of neurons could underlie the altered distribution and greater range of recorded FFI strengths (some with no FFI while the remainder trend towards abnormally strong values). If functionally mature GABAergic input from FS interneurons is indeed a crucial factor for supporting synaptic potentiation, one might predict that during single-cell TC plasticity experiments, the degree of potentiation achieved might form a similarly split population of cells. In TC LTP experiments reported by Harlow *et al.* (2010), although single-cell, perforated patch techniques were used, the recordings were performed in the presence of GABA<sub>A</sub> antagonists, thus such an effect cannot conceivably contribute to this conclusion.

**4.6.3. Comparison between changes to FFI in the present data and sensory deprivation studies**

Chittajallu and Isaac (2010) asked if the developmental normal integration of FS interneurons into layer 4 circuitry and emergence of FFI was impaired by perinatal sensory deprivation. To what extent do *Fmr1-KOs* overlap with sensory-deprived animals? Directly comparing P10-11 *Fmr1-KO* FFI strength data in the present study to age-matched whisker deprived animals presents a similarly mixed picture.

Differences between findings in the sensory deprived cortex by Chittajallu and Isaac (2010) and the results of the present study are summarised as follows: Firstly, they found that whisker trimming did not affect any properties of the local connection between FS interneurons and excitatory

41 In the visual cortex, only manipulation of GABAA α<sub>1</sub> receptor subunits – principally targeted by PV+ FS interneurons (Fritschy and Brünig, 2003) – is associated with shifted critical periods (Fagiolini et al., 2004).
neurons: connection probability, strength and release probability were unaffected by sensory deprivation. This is incompatible with results of the present study: in *Fmr1*-KOs FS-Ex connectivity was dramatically reduced compared to wild-type levels, and short-term depression of the unitary connection (as a proxy for release probability) was increased (Figures 3-20 and 3-21, respectively). Moreover, intrinsic properties of P9-11 FS and Ex neurons were unaffected by whisker trimming from birth, whereas both input resistance and membrane time constant were significantly enhanced in *Fmr1*-KOs (with intact whiskers) in the present study. However, the authors did find that trimming whiskers did affect the strength and effectiveness of TC FFI. Similar to the present study, some cells were observed without FFI, and average G/A ratios of cells with FFI were altered, but weaker rather than stronger as presented here from *Fmr1*-KO recordings. They explain this effect through demonstrating changes to the relative excitatory strength of thalamocortical input to FS vs. Ex neurons in paired recordings. Normally, a developmental increase in this ratio is observed between P6 and P11 that enhances the relative recruitment of FS interneurons, as well as a developmental downregulation of an Mg2+-insensitive NMDA conductance at TC inputs to Ex neurons. These findings were not examined in the present study, although further examining the decay kinetics of TC EPSCs in Ex. neurons (voltage clamped at -70mV where the NMDA conductance was observed in the previous study) suggests no change to a slow component of a dual exponential fit to decay kinetics (data not shown). Further work would be necessary to definitively characterise this however, since it is conceivable that some contamination
from slow kainate conductances could exist. This would provide a further source of charge transferred from TC input to Layer 4 Ex. neurons and boost synaptic integration over longer timescales. Finally, Chittajallu and Isaac (2010) showed that the overall effect of trimming led to a reduction in the G/A ratio by a relative decrease in the FF-IPSC strength onto the excitatory postsynaptic cell. A corresponding mean increase in EPSP half-width was observed that was predicted by both the cells’ G/A and NMDA/AMPA ratios at -70mV. In the present study however, an increase in the relative FF-IPSC/EPSC strength was observed in Fmr1-KOs. This change was found to be ineffective at constraining the EPSP half-width or paired-pulse summation for moderate G/A ratios (G/A=0-2), but over-effective for strong G/A ratios (G/A>2), with excessive hypo-summation observed during paired-pulse stimulation of VB. The previous study did not find any changes to FF-IPSC synaptic kinetics brought about by whisker trimming, however, in the present study the significantly slower IPSCs in Fmr1-KOs played a large role in contributing to altered FFI in these recordings.

Overall, changes to the FFI circuit presented here show distinct differences when compared to the perinatal sensory deprived state. Overall, the present data firmly implicate intracortical abnormalities in Fmr1-KOs, rather than confined to thalamocortical synaptic alterations as shown for whisker trimming experiments by Chittajallu and Isaac (2010).
4.6.4. Mechanisms contributing to stronger FFI run-down in *Fmr1-KOs*: thalamocortical vs. intracortical short-term plasticity?

In the present study, both direct thalamocortical and feed-forward inhibitory inputs to Layer 4 excitatory neurons show stronger and more progressive run-down in *Fmr1-KO* neurons during repetitive activation.

The greater disparity between the two rates of depression could arise from two sources, either alterations in the relative strength and STD at TC inputs to FS interneurons, or in the STD of intra-L4 unitary FS-Ex. connections. Unlike the situation at P14 and P28, where short-term plasticity at the FS-Ex. synapse is unaffected by the absence of FMRP (Gibson et al., 2008), it is clear that in the present study at P10-11 a contribution to the *Fmr1-KOs* phenotype indeed comes from stronger STD between FS and Ex. neurons (Figure 3.21c,d). Similarly, the effect of a significantly reduced FS-Ex connection probability in these animals (Figure 3.20) suggests that the redundancy in the feed-forward GABAergic projection is reduced. This might contribute to the observation that strong step-wise paired-pulse, rather than gradual run-down of FFI was more pronounced in *Fmr1-KOs* compared to wild-types. Taken together, these findings demonstrate a reduced stability of FFI in *Fmr1-KOs* that contributes detrimentally to the frequency sensitivity performance at TC inputs to Layer 4.

4.6.5. Mechanisms contributing to altered TC short-term plasticity

Individual thalamocortical axons are thought to target both FS and Ex neurons in Layer 4 (Cruikshank et al. 2007, 2010; Inoue and Imoto 2006;
although see Kimura et al. 2010). At the TC synapse however, while postsynaptic specializations such as Ca^{2+}-sensitive AMPARs largely explain the differential recruitment of FS and Ex neurons by thalamic input (Cruikshank et al., 2007; Hull et al., 2009), different presynaptic release probabilities are observed at TC inputs onto these two cell classes, such that STD is faster at inputs to FS than to Ex. neurons (Gabernet et al., 2005; Cruikshank et al., 2010). Studies of other central synapses which show target-specific release probability from boutons of a common axonal fibre demonstrates that this can arise via multiple mechanisms, ultimately constrained by presynaptic calcium buffering kinetics (Schneggenburger and Neher, 2000; Rozov et al., 2001; Atwood and Karunanithi, 2002).

These presynaptic mechanisms are diverse and therefore raise multiple potential targets for disruption by loss of FMRP, including specific expression of presynaptic metabotropic receptors (Scanziani et al., 1998; Dalezios et al., 2002), specializations in presynaptic VGCCs (Ali and Nelson, 2006), trans-synaptic molecules (Sylwestrak and Ghosh, 2012), and vesicle release machinery (Holderith et al., 2012; Narboux-Neme et al., 2012). The latter is of particular interest, since RIM1, involved in coordination of vesicle release machinery (Schoch et al., 2002) and essential for presynaptic LTP (Castillo et al., 2002), is expressed in thalamocortical axons and is a major target of FMRP (Iossifov et al., 2012). Despite having no reported effect on TC axonal anatomy loss of RIM1 leads to abnormal

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42 It is important to note here that release probability is variable as a function of network activity, and by extension, of experimental conditions (i.e. recording conditions in slices, and awake vs. anaesthetized in vivo preparations (e.g. Reig et al. 2006).

43 This is covered elsewhere (see Chapter 3), but it is noted that mRNAs coding for ~30% of the presynaptic proteome are affiliated with FMRP (Liao et al., 2008; Klemmer et al., 2011). Several reports have demonstrated altered short-term plasticity (Gibson et al., 2008; Deng et al., 2011, 2013; Testa-Silva et al., 2012), and notably, cell-type specific connection changes (Gibson et al., 2008; Patel et al., 2013).
TC axonal function and strikingly, aberrant developmental dendritic orientation of Layer 4 spiny stellate neurons (Narboux-Neme et al., 2012), as also seen in *Fmr1*-KO mice (Till et al., 2012). Moreover, RIM1 knockout mice are reported to show defective pre-pulse inhibition and “additional behaviours linked to Schizophrenia” (Blundell et al., 2010), implicating RIM1 in normal cognitive function.

The short-term plasticity of thalamocortical synapses also undergoes coordinated developmental changes during the first two postnatal weeks (Yanagisawa et al., 2004; Borgdorff et al., 2007; Zhang et al., 2011). It is conceivable therefore that changes in *Fmr1*-KOs seen in the current study could be influenced by a developmental lag. Interestingly however, Yanagisawa and colleagues (2004) report that paired-pulse depression at TC terminals (as a proxy for release probability) is sensitive to whether the synapse is postsynaptically silent or not. Tested between P4-P22, the authors find a developmental decrease in release probability during pharmacologically isolated NMDA-only postsynaptic responses, but a developmental increase for isolated AMPA-only responses. They go on to describe two classes of terminals, with high-release probability (putatively NMDA-only) connections decreasing during the critical period.

A mixed population of TC terminals raises the interesting possibility that some level of pre-postsynaptic (or post-presynaptic) crosstalk exists at individual synapses and plays an active role in plasticity during the critical period. It does however raise a somewhat circular argument as to whether the abnormally late silent synapses and critical period plasticity reported in their hands is a secondary effect of elevated release probability at
thalamocortical synapses. At the ages tested in this study, this is unlikely, since presynaptic expression of FMRP was not observed at P7 and TC paired-pulse ratios were normal at P4 and P7 in their hands. This is consistent with the finding that FMRP is expressed only postsynaptically at P7, but shows both pre- and postsynaptic localization (and influence) by P14 (Wijetunge, 2009; Till et al., 2012).

In light of this evidence, stronger STD at TC-Ex. synapses in P10-11 Fmr1-KOs is consistent with a presynaptic phenotype emerging at the developmental onset of presynaptic FMRP expression. For reasons discussed above however, it is unwise to generalise this phenotype to TC inputs onto FS neurons. In addition, while not presented in this thesis, no changes to STD of TC inputs to FS cells was seen in pilot recordings from Fmr1-KO slices (n= 3 animals of each genotype)\(^\text{44}\).

4.6.6. How can stronger FFI emerge from a circuit with statistically fewer connected feed-forward interneurons?  

Firstly, how large is the population of FFI-providing interneurons in the normal circuit? The model of FFI presented by Gabernet et al. (2005) is instantiated by connecting a population of 100 integrate and fire FS interneurons to a single postsynaptic Ex. neuron. This can be considered a realistic estimate: Hanno and colleagues painstakingly reconstructed the laminar distribution of all GAD67\(^{+ve}\) neurons in multiple barrel columns from one-month old rats and found an average number of 300~400 per

\(^{44}\) Comparison to older ages is not possible since Gibson et al (2008), did not test STD of this ascending connection at P14 to either Ex. neurons of FS cells.
layer, per column (Meyer et al., 2011). Of these, at least 55% were co-immunoreactive for PV (i.e. PV cells were the most highly co-labelled interneuron marker within Layer 4: Meyer et al. (2011), Figure S4).

However, Sun and colleagues (2006), used paired recordings to build a body of evidence suggesting that TC FFI is provided by one or a few FS interneurons. Indeed, it is possible to dramatically alter the strength and behaviour of FFI in one postsynaptic Ex. neuron by preventing a connected FS interneuron from firing by injecting hyperpolarising current (e.g. Figure 4-29a), in some cases abolishing FFI completely. Regarding the redundancy and non-specificity of FFI however - as with other studies (e.g. Gibson et al. 2008), these paired connections were tested in cells within 50µm of each other (putatively within the same barrel), using targeted patching based upon subjective experimenter choices based upon somatic morphology. Both these factors introduce sampling bias. Although presented as FS cells here for simplicity, in reality FFI is provided by diverse anatomical classes of basket-forming interneurons (Porter et al. 2001), some with barrel-confined projections, some with projections that span several barrels (Wang et al. 2004). It is possible that programs of functional development (that is, maturation of synaptic connectivity and intrinsic properties) of these individual cell classes are dissected by the loss of Fmr1, such that the maturation of connectivity from one sub-class is specifically or predominantly affected. Electrophysiological diversity of recorded FS interneurons is explored in Chapter 2 (methods) using cluster analysis techniques. Recent evidence showing sub-classification of Layer 4 FS interneurons is provided by Koelbl et al. (2013). Confirmed as connected to
Layer 4 Ex. cells, three distinct subgroups of FS cells could be distinguished by axonal and dendritic anatomy. In particular, one particularly highly connected subtype had projections that were tightly confined within barrel boundaries. Interestingly, this cell type was also found to provide inhibitory synaptic contacts that were outside the normal perisomatic domain. This is of particular interest with respect to the current data, since alterations to the synaptic anatomy of basket innervation provided by FS neurons could potentially underlie the longer evoked FF-IPSC decay kinetics found in *Fmr1*-KO recordings.

Using higher throughput connection sampling across a wider cortical area using optical mapping, it has been suggested that in comparison to Som⁺ interneuron-mediated cortical inhibition (e.g. Chiu et al. 2013, although see Fino and Yuste 2011, as well as Chapter 5 for discussion of differential functional recruitment), PV⁺ interneuron-mediated inhibition is dense and promiscuous (at least in Layers II/III and V from multiple brain regions), with connection probabilities higher than previously reported and constrained largely by axonal-dendritic overlap (Packer and Yuste, 2011).

This configuration supports robust FFI from at least one FS neuron, with the upper bound on number of recruited FS neurons dictated by factors including the number, position and axonal-dendritic overlap of the two cell classes, their probability of interconnectivity, their probability of shared connectivity to the same presynaptic thalamic afferent, and the extent of a shared presynaptic TC axon’s elaboration within the barrel. This estimate negates probability of synaptic failures for simplicity but it is acknowledged that the cohort of FS interneurons is likely strengthened by
multiple gap junction coupled cells acting \textit{in syncytia} during the first two postnatal weeks (e.g. Parker et al. 2009)

For the wild-type situation therefore, the total cohort of connected FS cells presynaptic to an Ex. neuron is a superset of those recruited by a thalamic axon exciting the Ex. neuron. In the \textit{Fmr1-KO} case however, unitary FS-Ex. connection strengths were unaltered despite the presence of both Ex. cells with no FFI and those with abnormally strong FFI. It is possible therefore that the number of recruited FS cells providing FFI is altered.

Ex. cells with no FFI are more easily accounted for, since it is conceivable that they have no connected FS neurons. Conversely, Ex. cells with stronger FFI might result from altered combinations of factors discussed in the previous paragraph. For example, abnormally diffuse axonal projections by FS cells, when combined with abnormally diffuse Ex. cell dendrites, and/or more diffuse thalamocortical projections could account for more widespread and statistically increased numbers of recruited FS interneurons and stronger FFI.

How biologically plausible is this model for anatomical disruption? From an anatomical perspective, the cytoarchitectonic structure of Layer 4 of the barrel cortex, undergoes three activity-dependent developmental milestones during the early postnatal period: Segregation of TCAs into whisker-related patches, segregation of Layer 4 stellate neurons into cell-dense walls and cell-sparse hollow, and the selective dendritic orientation of stellate neurons towards the emergent barrel hollow. These processes are sensitive to normal sensory input and rely heavily on glutamatergic
signalling (reviewed extensively in Erzurumlu and Kind 2001; Fox and Wong 2005; Li and Crair 2011; Erzurumlu and Gaspar 2012). In Fmr1-KOs, at the level of their compartmentalised ramification in Layer 4, TCAs do not appear to be abnormally diffuse: TC axons appear to respect normal barrel boundaries, as revealed by staining for expression of the serotonin transporter (5-HTT) expressed presynaptically on TC terminals in Layer 4 (Till et al., 2012). This is consistent with a previous report showing that both normal development as well as whisker-deprivation induced anatomical plasticity progresses as normal in Fmr1-KOs (Harlow et al., 2010). This does not exclude the possibility of aberrant TCA branching below Layer 4 or between barrels without terminals in the septae. However, despite finding no evidence of dendrites projecting towards inter-barrel septae or into adjacent barrels, Till et al. (2012) found that at P7, wall-hollow segregation of stellate neuron somata was slightly reduced in Fmr1-KOs. Furthermore, at P14, despite retaining a high degree if polarisation, the selective orientation of stellate cell dendrites towards the barrel hollow was altered in the knockout, at which time the wall-hollow segregation matched that of wild-types. Polarised dendritic orientation was found to be normal at P35. This is seemingly in contrast to a previous study (Galvez et al., 2003) documenting sepal dendritic projections in adult (P60) Fmr1-KO mice on two different congenic strains.

Whilst unlikely to be sampling VPM thalamic termini from the adjacent barrels, it is possible that these cells, by projecting dendrites into the surrounding inter-barrel space could have altered responsivity to paralemniscal projections from the thalamic PoM nucleus that typically
innervate this area and convey multi-whisker input, albeit forming few synapses in Layer 4. Galvez et al. (2003) presented no evidence that these extrabarrel dendrites conveyed functional input. Nonetheless, it will be important to examine the spatial extent and compartmentalisation of functional whisker responses in Layer 4, perhaps by examining uptake of 6-deoxyglucose or by imaging intrinsic optical or voltage-sensitive dye signals. This would also ascertain whether responses to surrounding whiskers are indeed affected. This could be investigated in vivo by studying the relative contribution of surround whisker responses in the principle barrel, which are known to affect synaptic integration of principle whisker responses in Layer 4 (Higley and Contreras, 2005b). A cautionary tale here however comes from Bureau et al. (2008), who found that in young Fmr1-KOs, despite possessing abnormally broad and diffuse axonal projections into Layer II/III, connection probability from Layer 4 was reduced – i.e. anatomy does not reliably predict function.

Do changes to FS interneuron axonal or dendritic arbours contribute to this hypothesis? Selby and Sun (Selby et al., 2007) investigated the quantitative laminar position of subtypes of interneurons (Calbindin-, Calretinin- and Parvalbumin-positive) in the Fmr1-KO barrel cortex. Through protein immunohistochemistry, they found a specific reduction in the overall number of PV-reactive neurons, strongest in Layer 4, but a relative increase in deeper layers. Furthermore, they found significantly enlarged somatic area in PV+ cells quantified from Fmr1-KO tissue sections. These effects were seen in aged mice (>1 year old) and were not visible in hippocampal
sections\textsuperscript{45}. Whilst dendritic or axonal anatomy was not evaluated, these findings do suggest that anatomical and positional changes to (putative) FS interneuron take place in the \textit{Fmr1-KO} mouse, thus it is conceivable that this could contribute to effects on FFI observed in the current study.

Finally, the authors show altered TrkB (BDNF sensitive) receptor immunoreactivity on \textit{Fmr1-KO} PV\textsuperscript{+} neurons. This is of particular relevance since BDNF signalling via TrkB receptors is known to stimulate FMRP expression in hippocampal cultures (Castrén et al., 2002), and BDNF mRNA is abnormally distributed and produced in these mice (Louhivuori et al., 2011). More broadly, BDNF contributes to the survival, morphological maturation and lifelong formation/plasticity of synapses (Gómez-palacio-schjetnan and Escobar, 2013; Park and Poo, 2013). Compellingly, increasing BDNF expression in interneurons causes accelerated dendritic elaboration, suggesting that it plays an autocrine signalling role in dendritic development (Wirth et al., 2003), in a similar manner as has recently been shown for its role in the regulation of axon path-finding and dendritic asymmetry (Cheng et al., 2011).

In summary, a potential explanation for why some \textit{Fmr1-KO} Ex. cells in the current dataset receive stronger FFI could be as a result of more widespread recruitment of FS interneurons. This could plausibly arise via more diffuse TC axonal projections, larger FS axonal fields or larger Ex. dendritic fields. One final possibility not discussed up to this point is the potential for a greater sensitivity of TC axons to stimulation (thus easing

\textsuperscript{45}In fact, one report demonstrated increased PV\textsuperscript{+} density in post-mortem human hippocampal tissue from Autistic patients (Lawrence et al., 2010)
the recruitment of a larger bundle with the same stimulation strength and easing the recruitment of FS neurons in neighbouring barrels - putatively connected but not normally recruited by TC inputs). While potentially artefactual to the brain slice preparation, stimulation of VB using a range of stimulation strengths showed a markedly steeper input-output curve for evoked amplitudes (data not shown), suggesting that TC fibres do indeed display altered electrophysiology in *Fmr1*-KO slices at this age. This is further supported by abnormal TC response latencies in *Fmr1*-KO recordings from both FS and Ex. neurons in Layer 4, putatively due to slower conduction velocity. This effect warrants further investigation.

Finally, the approach taken in this chapter has been to build a mechanistic understanding of a circuit dysfunction through combined and bidirectional physiology and modelling. By studying seemingly small pathophysiological changes in the component parts of a well-described dynamic circuit, complex emergent thalamocortical hypersensitivity could be predicted. This level of analysis will be essential to gaining further insight into the convergence of pathophysiology in disease mechanisms affecting circuit function. Moreover, as explored here for the sensitivity of abnormal circuit function to FFI modulation, this approach provides a powerful framework to support experimental manipulation of homeostatic resilience of canonical circuit computations in the face of targeted treatments.
5. Distorted representation of thalamocortical inputs by cortical network activity in the *Fmr1-KO* primary somatosensory cortex at P10/11
5.1. Key findings

Transiently sustained intracortical network activity was evoked by thalamocortical burst stimulation. Properties of network activity including individual neuron spike times and feedback currents and spatiotemporal propagation of network activity were studied. The main findings were:

1) Spiking activity of Layer 4 neurons during a period of network activity sustained by local circuit firing was disrupted in Fmr1-KO recordings. Spikes were fired later and at a lower rate, with lower overall temporal precision.

2) Whole-cell voltage clamp analysis of input currents arising during network feedback demonstrated stronger GABAergic feedback inhibition in Fmr1-KO recordings that was most likely due to integration of greater inhibitory charge relative to excitatory charge. The timing of feedback inhibition was slower and imprecise in Fmr1-KOs however.

3) Using a 64 channel multi-electrode array recording apparatus, properties of extracellular cortical signals were investigated.

   a. Layer 4 Local Field Potential (LFP) recordings showed a shorter time-course after thalamocortical stimulation in Fmr1-KOs, consistent with less prominent network sustained synaptic activity. Consistent with this interpretation, Layer 4 Multi-Unit Activity (MUA) demonstrated a substantially lower rate of firing activity in Fmr1-KOs.

   b. Spatiotemporal propagation of cortical network activity was investigated using 2D Current Source Density analysis of LFP signals, and Principle Component Analysis of MUA signals. Intracortical propagation of network activity was weaker in Fmr1-KO recordings, propagating a shorter distance and involving a smaller area of cortical tissue.

   c. Spectral analysis of LFP signals in Layer 4 showed an average increase in gamma oscillatory power during network activity in Fmr1-KO recordings, but a reduction in higher frequency (>100Hz) components associated with correlated extracellular spike waveform. Consistent with this interpretation, reduced centre-surround correlation was observed in Fmr1-KOs between MUA signals in Layer 4 and those on simultaneously active channels.

Together, these data show that intracortical network activity evoked by thalamocortical stimulation is abnormal in Fmr1-KOs at P10-11, with disorganised spiking activity and synaptic currents observed in Layer 4, and weaker overall sustained network activity. The circuit contributions to these phenotypes are examined.
5.2. Introduction

In the previous chapter, excessive sensitivity of Layer 4 Ex. neurons to high frequency TC input was demonstrated in recordings from \textit{Fmr1}-KOs. This led to a pronounced relaxation in the coincidence detection criteria at TC inputs to Layer 4, such that lower frequency TC pulse trains could evoke cortically sustained circuit activity.

A predominant component of this effect was from altered intrinsic properties: i.e. their increased input resistance and longer membrane time constants. Furthermore, in Chapter 3, widespread disruption to synaptic connectivity in Layer 4 was observed, with a strong contribution from intracortical sources as revealed by paired recordings and analysis of spontaneous currents. It is an easy prediction therefore, that circuit activity in \textit{Fmr1-KO} Layer 4 will be distorted by these changes. The aim of this chapter is to describe these changes, using the response to TC input to evoke a network response.

Spontaneously arising and TC-evoked “UP states”, transiently sustained bursts of intracortical activity, are longer in older (2-4 weeks old) \textit{Fmr1}\textsuperscript{-/}\textsubscript{Y} tissue (Gibson et al. 2008; Hays et al. 2011, although see Gonçalves et al. 2013), and implicate a reduction in inhibition/excitation balance. Conversely, in the present study of the P10/11 \textit{Fmr1-KO} TC circuit, \textit{elevated} FFI strength, but bidirectionally reduced intracortical excitatory-inhibitory connectivity was found. How do these phenotypes interact with elevated excitability in Layer 4 neurons to influence the intracortical representation of TC inputs by sustained network activity? In this chapter, these questions
are addressed by investigating TC-evoked intracortical activity. Here, using a battery of experimental and analytical techniques, changes to single cell spike patterns are demonstrated, supported by abnormal excitatory/inhibitory network inputs, leading to a widespread disruption in the spatiotemporal and spectrotemporal evolution of intracortical population activity.

5.3. Results

5.3.1. Experimental approach and considerations

5.3.1.1. Inferring network activity from single cell-attached recordings

To faithfully infer the properties of network-sustained spiking activity from the firing patterns of single or small numbers of participating cells, it is essential to only minimally influence the firing behaviour of neurons. Since whole-cell recordings dialyse the intracellular contents, introduce parasitic series resistance and capacitance (distorting membrane time-course), and bias the resting potential of recorded neurons, minimally invasive cell-attached recordings were adopted for these experiments (Perkins, 2006). Figure 5-1 demonstrates a representative example of this recording approach. Neurons could be maintained in this configuration for extended periods and firing responses were typically stable for >2.5 hours, albeit reducing the probability of successfully obtaining a high quality whole-cell recording after this duration.
Since 5x50Hz stimulation could reliably (>90% mean success rate) evoke intracortical network activity in both wild-types and Fmr1-KO recordings, this stimulus pattern was used by default to stimulate recurrent circuit activity in the Layer 4 circuit. Figure 5-1a shows the recording of this activity simultaneously in two Layer 4 Ex. neurons in a brain slice from a P10 wild-type animal. After the recording, whole-cells access was obtained and both cells were confirmed as receiving a direct TC input by the presence of a TC EPSC. Despite receiving TC input, cell 1 in this example recording did not fire during the direct TC stimulation period, only firing in response to network activity that outlasted the TC stimulation period by several hundred milliseconds. Conversely, cell 2 fired both in response to direct TC stimulation and during the lasting period of network activity. Whilst spike times were variable between trials, highest spike counts were observed within approximately 50-100ms of the last stimulus, reducing gradually over the subsequent ~100ms (Figure 5-1b).

5.3.1.2. Controlling for inter-slice variability

A potential confounding factor in this experimental approach is that inter-slice variability in the number or geometry of severed TC axons and intracortical tissue, or the position of the simulation electrode could affect the reproducibility of evoked circuit activity between brain slices. Furthermore, the anatomy or sensitivity of TC axons to electrical stimulation could be different between the two genotypes, or the anatomy of the neurons supporting the recurrent circuit activity in Layer 4 could be altered in the Fmr1-KO animals.
Figure 5-1 Experimental approach for quantifying TC-evoked network activity in Layer 4

a). Left: Schematic recording configuration showing cell-attached recordings from a pair of Layer 4 Excitatory neurons with Layer 4 network activity evoked by TC stimulation with 5x50Hz pattern. Right: example paired recording in a slice prepared from a P10 Fmr1<sup>+/Y</sup> animal, showing 500 overlaid trials. (20s inter-trial period) Spikes are observed as cell-attached action currents. Overall success rate (where either cell fired at least 1 spike) was >90%.

b). Responses were extremely stable for nearly 3 hours of repeated trials. Spike times (blue crosses) and Post-stimulus time histograms for both cells (below, 5ms bins). Note that cell 2 typically fired both during the stimulus train and for a protracted period after the end of stimulation, while cell 1 participated in network activity, but did not fire during TC-evoked stimulation.
a

Cell-attached

5x50Hz stim. (500 trials overlaid)

Cell 1

Cell 2

VB
thalamus

5x50Hz

b

Cell 1

Cell 2

Post-Stimulus Distribution of Spike Latencies (ms)
It is thus conceivable that these and other sources of under-controlled variability could distort the response properties of circuit activity, including the rate and timing of AP firing. However, it would be difficult to parse which are biological effects of genotype, and which are experimental artefacts, particularly since a prediction from data in the previous chapters is that disruptions to rate and timing of cortical network activity are likely in Fmr1-KO recordings. One strategy could be to adjust stimulation strength such that the first TC-evoked spike time, or the total number of evoked spikes was comparable between slices. This would be biologically unrealistic however, due to the observation of strong within-slice variability in the participation of Layer 4 Ex neurons in recurrent circuit activity (e.g. see Figure 5-1). Indeed, a previous study found that above a threshold, the frequency of TC stimulation, not the intensity was the critical factor in evoking a cortical network response, with further increases in stimulation strength largely ineffective at altering the response properties (Beierlein et al., 2002). In the previous chapter, an altered frequency sensitivity of TC responses in Layer 4 was demonstrated in Fmr1-KOs using recordings in which neither the stimulation strength or TC-evoked EPSC or EPSP amplitudes were significantly different between genotypes. Due to the increased number of trials carried out in recordings for this chapter, to avoid damaging TC fibres, here VB stimulus intensity was adjusted to the minimum necessary to evoke a spiking response in >60% of trials for each recording. No significant effect on either the number of spikes fired, or the timing of the first spike in successful trials was observed by further increasing stimulus intensity for either genotype (an
example recording is shown in Figure 5-2). However, since the aim of this chapter is to quantify potential change network-sustained activity, to further reduce the impact of altered TC-evoked summation effects, a 200ms window of analysis approach was adopted, where only spikes that occurred >20ms after the last TC stimulus were analysed. Where analysis of the whole peri-stimulus spike train (rather than data confined to after the end of stimulation) is included, it is noted explicitly in the text and figure legends. Despite trying a number of approaches such as analysis of input currents by template deconvolution or post-stimulus latency, separating feedback, network-driven excitation from excitation driven by direct TC stimulation remains intractable within the limits of this study.

5.3.1.3. Minimal thalamocortical participation in intracortical network activity

A further consideration is the possibility that recurrent TC oscillations contribute to network activity recorded in the cortex. As discussed above, this has been reported in vivo during the first postnatal week (Minlebaev et al., 2011). Spindle-like TC oscillations have been pharmacologically induced in slices preparations (Tancredi et al., 2000), but notably require substantially thicker (650μm) slices, suggesting that more intact TC connectivity is required. Furthermore, these burst were not visible in the absence of pharmacological stimulation, even with repetitive TC or white matter stimulation. Thus it is most likely that the network activity discussed in this chapter is sustained by intracortical firing, rather than through TC oscillations.
Figure 5-2 Effect of TC stimulus intensity on network activity in Layer 4 as revealed by spike firing statistics

a). Spike times from a P10 Fmr1<sup>−/−</sup> cell-attached Layer 4 Ex neuron recording, showing stereotypical firing pattern that was largely unaffected by varying the VB stimulation strength from 50-250μA.

b). Spike firing statistics for cell shown in a). Data show mean±SEM for 40 trials at each stimulus intensity, with 15s inter-trial period, sampled in pseudo-random order. Note that increasing stimulation intensity >150μA produces little additional change to firing statistics.
Trials ordered by pseudo-random sampling order

Trials ordered by stimulus intensity

Total spike counts

(a)

(b)

No. spikes evoked /trial

1st spike latency (ms)

Firing probability

Stimulation strength (µA)
Nevertheless, increased neuronal excitability in \textit{Fmr1-KOs} could alter this behaviour: routinely, field electrodes were placed in VB or RTn during experiments and rhythmic oscillatory activity was not observed. This conclusion is in agreement with work from the Yuste lab, who reported no suprathreshold summation or polysynaptic activity in thalamic or RTn neurons when CT axons were stimulated at frequencies that evoked UP states in the cortex (Beierlein et al., 2002). Furthermore, MacLean et al. (2005) reported that TC-evoked and spontaneous cortical UP states were nearly identical, and severing TC projections had little effect on spontaneous cortical population activity. Therefore, network activity reported in this section is considered evoked by TC input but sustained by intracortical firing.

5.3.2. Abnormal timing of TC-evoked network activity in Layer 4 in \textit{Fmr1-KOs}

Cell attached recordings were made from Layer 4 Ex neurons in slices which showed Layer 4 field potential responses to TC stimulation, (this could be confirmed with subsequent whole-cell access or simultaneous recording from an adjacent cell). At the response probability threshold of >60%, 15 trials typically collected >10 in which spiking activity was observed. Example recordings from wild-type and \textit{Fmr1-KO} neurons are shown in Figure 5-3a. In these neurons, the \textit{Fmr1-KO} cell fired fewer spikes per trial and typically fired with a longer inter-spike interval compared to the wild-type example. The \textit{Fmr1-KO} example also fired earlier, responding after the second TC stimulus, with a peak spike count observed before the end of the stimulus train.
5.3.2.1. Shifted distribution of TC-evoked spike times, but not spike numbers in Fmr1-KOs

The proportion of tested neurons that responded with at least one spike during 15 trials are presented in Figure 5-5 with reference to their thalamocortical inputs, as examined after breaking in and obtaining whole-cell access. Interestingly, a slight increase in the fraction of neurons participating in network activity showed a slight increase in Fmr1-KO recordings. However, to examine the contribution of individual Ex. neurons to Layer 4 network activity, only neurons that fired during recordings are included in this analysis.

Firing patterns from wild-type and Fmr1-KO neurons are presented in Figure 5-3a-b and compared Figure 5-4. Firstly, the median number of spikes fired by Fmr1-KO neurons per one-second trial was not significantly different from wild-types (Figure 5-4ai).
Figure 5-5 Probability of successfully evoking a spiking response with TC stimulation: Proportion of cell-attached recordings from Layer 4 Ex neurons in which cells responded with at least one spike in at least one of 15 repeated trials with 5x50Hz TC burst stimulation.

Analysis is restricted to recordings from barrels in which network activity could be evoked with TC stimulation, either from non-stimulus–locked whole-cell or field potential activity. Below: In a subset of the above recordings, whole-cell access was subsequently obtained. Of these neurons, the majority received direct TC input, as resolved by voltage-clamp recording at the stimulation strength used to evoke circuit activity before breaking in. However, neurons in which no direct input from VB could be resolved were also observed to participate in network activity.
All cell-attached recordings

Recordings with break-through whole-cell access

% of neurons firing 1< AP

Confirmed VB-connected

Confirmed VB-unconnected
Figure 5-3 TC-evoked spike times in wild-type and *Fmr1-KO* Layer 4 neurons

a). **Top:** Example recordings showing network sustained firing activity evoked by thalamocortical burst stimulation at 50Hz (orange markers). Grey shaded regions indicate 200ms windows of analysis used in subsequent analyses to quantify spikes during network-sustained activity to reduce contribution from direct thalamocortical inputs; 15 consecutive trials (0.07Hz) showing trial-trial variability in the timing of spikes recorded in cell-attached configuration. **Bottom:** Spike count histograms (5ms bin width) for above neurons.

b). Mean spike probability density functions (5ms kernel standard deviation) for 21 *Fmr1*+/− and 16 *Fmr1*−/− neurons (maximum of 3 per animal).

c). Expanded view of spike probability density for neurons shown in b), ordered from top to bottom by time of first spike onset.
Figure a: Comparison of Fmr1<sup>+/Y</sup> and Fmr1<sup>−/Y</sup> trials.

Figure b: Spike density (spikes/s) over post-stimulus time (ms) for Fmr1<sup>+/Y</sup> (left) and Fmr1<sup>−/Y</sup> (right).

Figure c: Heatmap of cell number vs. post-stimulus time for Fmr1<sup>+/Y</sup> (left) and Fmr1<sup>−/Y</sup> (right).
Figure 5-4 Disrupted spiking patterns during recurrent circuit activity in Fmr1-KO Layer 4 Ex neurons.

Quantification of spiking activity from cell-attached recordings (n.b this analysis rejects cells that were silent during 1s peri-stimulus period). Number of neurons analysed in this dataset: Fmr1++/Y = 21, Fmr1++/Y = 16, maximum of 3 from each animal.

a). i) Across multiple trials, the average number of spikes fired during one second of TC-evoked network activity was not significantly different between responding Fmr1++/Y and Fmr1++/Y neurons (Mann-Whitney, p=0.6). ii) Fraction of total spikes fired during TC stimulation vs. during a 200ms post-stimulus window of analysis, for individual neurons. Fmr1++/Y neurons fired spikes in similar proportions during both periods on average, while Fmr1++/Y neurons fired a significantly higher proportion in the later period (Paired ratio t-test, p<0.01) (Extra sum-of-squares F test vs. null hypothesis of equal proportions in both periods (i.e. vs. slope = 0, grey): Fmr1++/Y: p=0.08, F=3.24; Fmr1++/Y *p<0.001, F=33.5).

b). Probability density for spikes fired by responding Layer 4 Ex neurons during the peri-stimulus period of TC-evoked network activity: mean±SEM spike density, calculated using 1ms time bins and spikes convolved with ±5ms St. Dev. Gaussian kernels. Peak mean spike density was reduced in Fmr1++/Y recordings, calculated across the whole 1s post-stimulus sampling period (t-test, p=0.008, mean±SEM spike density: Fmr1++/Y: 0.023±0.002, Fmr1++/Y: 0.015±0.0016 spikes.s^{-1}). Mean spike density averaged across the late 200ms window of was not significantly different between genotypes (t-test, p=0.7).

c). Spike rate and timing during a 200ms window of feedback-sustained network activity was significantly different in Fmr1++/Y recordings compared to Fmr1++/Y littermates: Bursts started later and lasted longer, with cells firing at rates that were slower and more variable between trials. Plotted points are individual neurons, bars show mean±SEM values. Asterisks indicate statistically significant differences between genotypes (p<0.05, t-test).
**Figure a**

Cumulative fraction of spikes for **+/Y** (blue) and **-/+Y** (red) conditions.

**Figure b**

Spike density vs. post stimulus time. The inset highlights the differences between **+/Y** and **-/+Y** conditions.

**Figure c**

Box plots showing significant differences in latency to first spike, burst length, spike rate, and rate jitter (CoV) between **+/Y** and **-/+Y** conditions.
To estimate the proportion of spikes fired as a result of the direct TC stimulation vs. during the feedback sustained activity, each neuron’s total spike count across 15 trials was split into the fraction of spikes falling before and after 100ms from the first TC stimulus. Wild-type neurons fired an approximately equal fraction in both periods, with no statistical difference in the average ratios between groups (Figure 5-4a). Conversely, Fmr1-KO cells fired a significantly greater proportion of spikes in the later period. Overall however, the median number of spikes fired within the feedback window was unchanged (1 for both genotypes – data not shown).

Given the previously discussed hypothesis that the Layer 4 circuit acts to amplify and prolong the response of TC inputs (Li et al., 2013a, 2013b; Lien and Scanziani, 2013), it is tempting to speculate that the relative contributions of TC and recurrent circuit inputs to Layer 4 Ex. neurons might be altered in young Fmr1-KOs. However, it is impossible to draw a conclusion using this experimental approach since both the decay kinetics of TC EPSPs, and the intrinsic membrane kinetics of the Ex. cells themselves are slower in Fmr1-KOs, thus it is conceivable that spikes arising through TC drive are delayed by several milliseconds and potentially contribute a greater fraction of total spikes to the later window of analysis.
5.3.2.2. Reduced spike TC-evoked spike density in recordings from *Fmr1-KO* excitatory neurons

Spikes fired by Layer 4 Ex. neurons in these recordings were variable between trials, sparse, (typically on the order 1~5 spikes over several hundred milliseconds), and formed heavy-tailed ISI distributions, indicative of rapid local temporal modulation. Therefore, directly comparing spike time histograms between neurons (and ultimately genotypes) would be relatively uninformative, with a trade-off presented by the choice of bin size between temporal accuracy (small bins) and comparative power (large bins). To address this issue, a probabilistic approach was taken whereby spike probability density was estimated from spike times (Szucs 1998). Briefly, each spike time is discretised to 1ms and convolved with a Gaussian probability density function with a sigma of 15ms. Each spike’s total integral is adjusted to 1 across a range of ±3 sigma. Correspondingly, spike times were smoothed such that a spike occurs at time t±30ms with 95% certainty, and contributes an extra ~0.1 spike density to that of next spike if it arises 30ms later. The resulting spike density convolution is a continuous distribution rather than a discrete train; more amenable to comparison between trials and recordings, and less sensitive to binning artefacts than with a raw spike count histogram approach.

Mean±SEM\textsuperscript{46} spike density for wild-type and *Fmr1-KO* neurons that fired following TC stimulation are shown in (**Figure 5-4b**). For wild-type

\textsuperscript{46} Bootstrapped 95% confidence intervals were also estimated by resampling spike density functions and despite being in good agreement with the standard error, are likely underpowered, a better approach would be to shuffle spike times within a window of analysis – this would require more data for high confidence. Standard error is therefore reported.
recordings, a sharp peak in spike density occurred at approximately 90ms after the first stimulus, before declining with an ~100ms shoulder. Over the whole 1s recording period, mean peak spike density was significantly reduced in Fmr1-KOs, predominantly through a reduction in the early (~90ms) peak such that the shoulder effect merged into a prolonged plateau of elevated spike density. The final decay phase of the Fmr1-KO spiking response followed approximately the same rate, but lagged that of the wild-type average spike density function slightly. Mean spike density was not significantly different during the 200ms feedback window however.

Together, these results suggest that the TC-evoked firing response of Layer 4 Ex. neurons is slower in Fmr1-KOs, and that disruption to firing patterns could emerge from altered spike timing rather than changes to the overall number of spikes.

5.3.2.3. Altered firing patterns by P10-11 Layer 4 Fmr1-KO Ex. neurons

In the barrel cortex, both the rate and timing of spikes are informative for sensory processing (Ahissar et al., 2000). Notably, Panzeri and colleagues (2001) found that estimating stimulus location from cortical firing patterns using a model containing both spike times and total number of spikes increased information content by 44% compared to one containing just spike counts. They also show that spikes arising early after touch onset are crucial to this property and conclude that precise spike timing is essential
for the fast, information-rich representation of sensory input by a small numbers of spikes.

Given the importance of precise spike timing in barrel cortex for sensory coding, individual and inter-spike times were investigated from cell-attached recordings to ask directly whether TC-evoked spike times were disrupted in Fmr1-KOs.

Again, analysis was restricted to network-sustained firing activity arising after the cessation of TC stimulation to reduced contributions from altered TC integration. In the analysis window, the first spikes fired by Fmr1-KO neurons were significantly later by nearly 20ms on average compared to those in wild-type neurons (Figure 5-4b).

Furthermore, the last spike typically occurred disproportionately later in the window (data not shown) in recordings from Fmr1-KOs such that the mean burst length (time from first to last spikes) was significantly longer. Overall mean spike rates, calculated from the reciprocal of inter-spike-intervals were significantly slower in Fmr1-KO recordings. Finally, both the timing of the first spike in the window (data not shown), and the mean spike rate were significantly less stable between multiple trials in Fmr1-KO neurons, as shown by elevated standard deviation and coefficient of variation, respectively.

Together, these data show that both within individual and between repeated stimulus presentations, spike timing precision is affected and firing fidelity is substantially reduced in the young Fmr1-KO Layer 4 circuit. These changes are expected to severely impair the faithful
representation of sensory input and internally generated network activity. Additionally, unreliable spike timing is expected to impact upon mechanisms underlying the maturation of functional connectivity by impairing forms of synaptic plasticity that rely on the detection of precise and repeated pre- and postsynaptic firing correlation, such as STDP. This is of particular relevance for the postnatal development of barrel cortex circuitry: Egger and colleagues demonstrated an STDP-dependent rule for the maturation of connectivity between pairs of Ex. neurons in Layer 4 at P14, and elucidated both pre- and postsynaptic mechanisms (Egger et al., 1999). The authors reported that when presynaptic and postsynaptic neurons fired within 25ms of each other, as few as five APs at 10~20Hz were sufficient to drive synaptic depression. They argue that by down-regulating the excitability of the recurrent Layer 4 circuit in response to highly correlated inputs during strong sensory stimulation, such a mechanism is capable of providing a dampening effect on the feed-forward amplification of thalamocortical responses. They also note long-term plastic changes in the neonatal cortex could arise from brief periods of synchronised low frequency neuronal activity.

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47 Subsequently using a similar timing-based paradigm in visual cortex Layer 4, Sáez and Friedlander (2009) reported both LTP and LTD between connected excitatory neurons, the sign of which was largely predicted by the release probability prior to pairing. Furthermore, Wang et al. (2012) showed that both the sign and mechanisms of plasticity at this synapse are developmentally regulated. Neuromodulation is likely an additional key factor in modulating STDP in this circuit – reviewed in (Pawlak et al., 2010).
5.3.3. Synaptic correlates of disrupted spiking activity by Layer 4 neurons in *Fmr1-KOs*

Altered firing patterns of Ex. neurons participating in Layer 4 network activity, as demonstrated in the previous section could arise from several, possibly simultaneously contributing scenarios. Firstly, the slower intrinsic membrane time constants of Layer 4 Ex. neurons could slow their responsiveness to synaptic input from network feedback currents, as was demonstrated in the previous chapter for feed-forward TC voltage responses. This is likely to account for a significant fraction of the slower firing rates and later spikes observed during network activity in this chapter. Secondly, weaker or reduced connection probability between Layer 4 Excitatory neurons, as supported indirectly by the lower rate of spontaneous EPSCs shown in Chapter 3 would rapidly degrade the recurrent circuit architecture of the Layer 4 network (as explored in Ashby and Isaac 2011). Intuitively, this might be predicted to reduce the duration of the intracortical network response, as fewer postsynaptic neurons would be engaged by the firing of a single presynaptic cell. However, the interaction between these and the effects of elevated intrinsic excitability as shown in Chapter 3 are hard to predict. Furthermore, lacking information on the degree of network heterogeneity, known to be critical for diverse emergent network properties (Litwin-Kumar and Doiron, 2012), a network modelling approach would be dangerously under-constrained by the current data, thus capable of making only weak predictions. Obtaining this data and biologically realistic network modelling, however, is the subject of ongoing work in *Fmr1-KOs* at the current time.
Finally, a significant contribution to disrupted firing patterns during network activity could arise through altered synaptic input during network feedback. This hypothesis is strongly supported by the functional anatomy of Layer 4: the recurrent circuit architecture is expected to rapidly propagate the abnormal spike times shown in the previous section among excitatory and inhibitory postsynaptic target neurons. Consequently, the firing pattern of each of these cells will in turn be affected, likely compounded by additional intrinsic and synaptic abnormalities as demonstrated in individual cells in Chapter 3, before projecting synaptic input either directly or indirectly back onto the first neuron. Furthermore, in Fmr1-KO animals as tested at P10-11 in this study, this effect is also predicted to alter the recruitment of inhibitory interneurons, potentially altering the balance of inhibitory/excitatory synaptic input projected back onto Layer 4 neurons during network activity. As shown in Chapter 3, reciprocal FS-Ex. neuron connection probability was dramatically reduced in Fmr1-KOs, while FS intrinsic excitability was increased. Although not presented in this thesis, non-FS (putatively Som+) interneurons also showed a trend towards reduced probability of connection to Ex neurons in Fmr1-KOs, as well as significantly elevated intrinsic excitability (the latter is presented as part of Chapter 2: discussing cluster analysis of Layer interneurons from blind recordings). Since non-FS neurons provide a substantial source of feedback inhibition in Layer 4 (Beierlein et al., 2009), this can be considered a significant target for further investigation that is outwith the aims of the current project. Nonetheless, FS interneurons do receive network feedback inputs, and fire APs during Layer 4 network
activity (see Figure 4-28). However if recently activated as part of a TC FFI response, their synaptic release onto Ex. cells is likely to be strongly depressed, and to a greater extent in Fmr1-KOs due to exaggerated STD at this synapse.

This will be unavoidably detrimental to the performance of a circuit relying on a high precision temporal code. Indeed, Houweling and Brecht (2008) showed that targeted stimulation of single neurons in rat barrel cortex (albeit in Layer 5b) could alter the behavioural report of animals trained to make whisker-mediated decisions. This finding contributed evidence suggesting that the cortical code supporting the representation of sensory information is sparser than previously recognised. The mechanisms by which this single cell manipulation affected cortical dynamics are obscure however, and like involved multiple feed-forward and feedback motifs of propagating network activity. More recently, London and colleagues extended this methodology to follow the downstream consequences of artificial single neuron stimulation on network activity (London et al., 2010). The authors found that by simultaneously monitoring local network activity with a multisite silicon shank probe, as a consequence, on average 28 extra spikes were fired by local neurons. Although the authors demonstrate very that precisely timed spikes are rare in their results, and argue that their data is more representative of chaotic rate coding, they nevertheless find that elevated network firing resulting from the ectopic firing of one single spike lasts for nearly 100ms at their extracellular spike detection threshold. This is a behaviourally significant duration: for example, for trained head-fixed mice participating at expert level in
whisker-dependent object location tasks, the latency between first whisker-object touch contact and the completion of a motor behaviour (e.g. licking for reward) can be as little as 100ms (e.g. Petreanu et al. 2012), during which time the animal’s brain must detect the contact with a pole, determine its location from sensory and proprioceptive inputs, make a decision to whether or not the pole is in a reward associated position, before making and finally executing a motor plan to lick for a reward. Disrupted activity at the level of S1 is likely therefore to significantly impair this processing chain.

5.3.4. Altered inhibitory/excitatory balance and reduced fidelity of currents during network-sustained synaptic activity in Fmr1-KOs

To ascertain whether altered timing or In/Ex balance of synaptic input contributes to the abnormal Fmr1-KO network activity shown in the previous section, excitatory and inhibitory polysynaptic currents were investigated during TC-evoked network activity in Layer 4. After obtaining the times of spikes fired during TC-stimulated network activity in cell-attached configuration, whole-cell access was attempted in a subset of cells. High quality (R\textsubscript{access} < 25MΩ) recordings were obtained using Cs\textsuperscript{+}-based internal solution containing the sodium channel blocker QX-314 to improve space clamp and aid the isolation of compound excitatory and inhibitory synaptic inputs. This is particularly important during evoked cortical “UP states” in slice the preparation (Shu et al., 2003), which are reminiscent of high conductance states typical of in vivo cortical network activity under conditions of slow wave sleep and anaesthesia (reviewed in Destexhe et al.)
Figure 5-6 provides an example of this process in a recording from a P10 wild-type neuron.

![Figure 5-6](image)

**Figure 5-6** Example recording showing the synaptic currents underlying network-sustained firing of a Layer 4 Ex. cell (P11, *Fmr1<sup>+/Y</sup>).

**Top:** 40 trials of cell-attached firing driven by 5x50Hz TC stimulation. **Bottom:** After breaking in, the neuron was dialysed with Cs<sup>+</sup>-based internal solution and synaptic EPSCs and IPSCs were isolated in voltage clamp. Grey traces show 10 individual trials, coloured traces show mean responses. Holding currents are shown offset for clarity.

Note that in contrast to the tightly stimulus-locked TC-evoked currents, high variability was observed in the timing and amplitude of network-evoked currents between successive trials.

**Figure 5-7a** shows example voltage-clamped synaptic currents from TC-evoked network feedback in wild-type and *Fmr1-KO* Ex. neurons. Again, note the delayed emergence of polysynaptic activity that was variably timed between trials, and additionally the strong short-term depression of
direct TC EPSCs and FF-IPSC in the *Fmr1-KO* example. The strong inter-
trial variability in the timing and amplitude of network-evoked feedback
currents confounded their analysis. It was not possible to record inhibitory
and excitatory synaptic inputs simultaneously \(^{49}\), and using paired
recordings to simultaneously record at both NMDA/AMPA and GABA\(_A\)
reversal potentials (e.g. Okun and Lampl 2008) was considered
incomparable between genotypes, given that the large changes in
inhibitory synaptic connectivity ratios seen in *Fmr1-KOs* could cause a
disproportionate reduction in the fraction of shared feedback inhibitory
inputs between neighbouring Ex. neurons \(^{50}\).

Furthermore, the network responses were resolved as large, compound
polysynaptic bursts, and were resistant to a variety of peak finding,
template matching and deconvolution approaches (see Methods for
strategies tried). For these reasons, analysis presented here focuses on the
average excitatory and inhibitory current envelopes, which lose the
majority of fast temporal information (compare grey and black traces in
**Figure 5-7a**) \(^{51}\), but retain the kinetics of the overall current envelope
modulation. From these average current waveforms, the mean feedback
G/A ratio was calculated for individual neurons by dividing the sample-
by-sample mean current, analogous for the procedure used to calculated
G/A ratios using peak amplitudes of FFI currents in the previous chapter.

**Figure 5-7b** demonstrates this approach for the neurons of both genotypes

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\(^{49}\) Rapidly switching between the two reversal potentials was tried, as proposed by Cafaro
and Rieke (2010), but was not fast enough without yielding large amplitude and
predominantly uncompensatable capacitive artifacts.

\(^{50}\) Indeed, cross-correlation between simultaneously recorded IPSCs in neighboring Ex.
neurons shows a trend in this direction in a small dataset – data not shown.

\(^{51}\) Surrogate EPSC–IPSC pairs of shuffled trials were examined, but were rejected due to
the disproportionate increases in trial-trial current variability observed in Fmr1-KO IPSCs.
shown in Figure 5-7a. Note again the rapid rundown of FFI from an initially stronger tone in the Fmr1-KO example. Also not the prolonged duration of feedback current activity of both EPSCs and IPSCs for this example neuron, compared to the wild-type example. As for the analysis of feedback spiking activity, a 200ms window of analysis (shaded in grey) was placed 20ms after the last TC stimulus to minimise contributions from direct TC-evoked currents.

Network-driven feedback excitatory and inhibitory synaptic inputs arising during this 200ms window are compared between genotypes in Figure 5-8, both as individual current components and ratiometrically.
Figure 5-7  Example excitatory and inhibitory feedback currents and mean GABA/AMPA ratios for recordings from representative wild-type and Fmr1-KO Layer 4 neurons.

**a).** 10 overlaid trials (grey) showing network activity evoked by 5x50Hz TC stimulation. Mean responses are shown in black for each cell. For clarity, holding currents are shown offset and traces are filtered. **b).** Mean G/A ratio as a function of time for cells shown in **a)**, calculated by dividing IPSCs by EPSCs with 1ms resolution. Orange triangles are stimulus markers; grey shaded regions indicate the 200ms window used for subsequent analysis of feedback activity.
Fmr1\textsuperscript{+/Y}

Fmr1\textsuperscript{-/Y}

G/A ratio

Post stimulus time (ms)

100pA

500ms
Figure 5-8 Altered properties of network-sustained feedback synaptic activity in P10-11 Fmr1-KO Ex. cell recordings. Data in this figure are from 10 Fmr1+/Y and 11 Fmr1−/− animals (1 cell per animal). Points are neurons, bars (or shading) indicate mean±SEM.

a). Mean±SEM GABA/AMPA current ratios during feedback activity were elevated and sustained in Fmr1−/− recordings, compared to wild-type littermates. Asterisk indicates statistically significant increase in the population mean G/A, average across the 200ms window, as expanded in b). Grand average G/A ratios during the 200ms feedback activity window were significantly elevated in Fmr1−/− neurons (t-test, p=0.04, GABA/AMPA ratio = 0.73±0.24 (Fmr1+/Y) vs. 1.65±0.32 (Fmr1−/−)). c). Charge transferred by voltage-clamped EPSCs and ISPCs during the 200ms window expressed as a ratio. Left: actual values of current integrals for each cell, showing linear fits, dotted line shows unity. Right: Charged transferred by each current component as a fraction.

<table>
<thead>
<tr>
<th>Charge (nC)</th>
<th>Fmr1+/Y (mean±SEM)</th>
<th>Fmr1−/− (mean±SEM)</th>
<th>p value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPSC</td>
<td>16.4±2.5</td>
<td>20.8±3.2</td>
<td>0.30</td>
</tr>
<tr>
<td>IPSCs</td>
<td>13.3±4.0</td>
<td>26.5±6.0</td>
<td>0.09</td>
</tr>
<tr>
<td>ratio</td>
<td>0.88±0.26</td>
<td>1.45±0.30</td>
<td>0.17</td>
</tr>
</tbody>
</table>

d). As in c), but plotting the trial-to-trial variability (standard deviation across 15 trials) in each current component individually (left), and as a fraction (right). IPSC variability, but not ESPC variability was significantly elevated in Fmr1+/Y recordings.

<table>
<thead>
<tr>
<th>Charge st.dev. (nC)</th>
<th>Fmr1+/Y (mean±SEM)</th>
<th>Fmr1−/− (mean±SEM)</th>
<th>p value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPSC</td>
<td>6.36±1.6</td>
<td>11.2±2.3</td>
<td>0.13</td>
</tr>
<tr>
<td>IPSCs</td>
<td>5.54±1.8</td>
<td>15.3±3.7</td>
<td>*0.03</td>
</tr>
<tr>
<td>ratio</td>
<td>1.11±0.2</td>
<td>1.57±0.2</td>
<td>0.08</td>
</tr>
</tbody>
</table>

e). The mean cross-correlation between mean EPSC and IPSC current envelopes was significantly reduced in Fmr1+/Y recordings compared to littermates (t-test, p=0.006, Pearson correlation coefficient = 0.72±0.05 (Fmr1+/Y) vs. 0.39±0.08 (Fmr1−/−)). Note that the timing of peak mean EPSC-IPSC correlation lags further by approximately ~30ms in Fmr1+/Y recordings. f). Charge transferred by mean feedback EPSCs and IPSC envelopes expressed as cumulative integrals, normalised to the total charge transferred in the 200ms window. A trend towards disproportionate rate of inhibitory charge integration, relative to excitatory charge integration was observed in Fmr1+/Y recordings, compared to littermates. Each line shows the charge integration balance of an individual neuron.
**Integrated feedback charge**

(c) 

**Integrated charge variability**

(d) 

**Fraction of total charge in window**

(f)
Strikingly, the mean feedback G/A current ratio was significantly elevated in *Fmr1*-KOs (Figure 5-8a and b). Furthermore, whereas in wild-types, the mean ratio diminished during the 200ms window form ~2 at the start to <1, in *Fmr1*-KOs the mean G/A ratio was greater than 1 for the entire duration of the 200ms window.

Interestingly, while the mean G/A current ratio was significantly elevated in *Fmr1*-KO recordings, the mean In/Ex charge balance was not significantly different between genotypes, despite a trend towards an increase (Figure 5-8c). Indeed, treated separately, changes to mean EPSC and IPSC charge integrals were disproportionate; IPSCs showed a strong but sub-significant (p=0.09) change in mean charge transferred between genotypes, whilst EPSCs were largely unaffected.

This suggests that in addition to changes to their relative strength, a change in the relative timing of the mean EPSC and IPSC current envelopes could contribute to the altered G/A current balance. Supporting this hypothesis, when the variability in inhibitory and excitatory charge transferred between repeated trials was compared (Figure 5-8d), strong but unbalanced increases were observed for both. In particular, a significant increase in trial-to-trial IPSC variability was found in *Fmr1*-KOs. Overall, when the covariance in trail-to-trial variability of feedback IPSC and EPSC charge was examined within-cell, these were disproportionate such that a moderate (p=0.08) increase in the In/Ex variability ratio was observed.
To more directly evaluate the relative timing of feedback EPSCs and IPSCs, mean current barrages were independently normalized by amplitude\textsuperscript{54}, and their cross-correlation was calculated (Figure 5-8e). Peak correlation between the EPSC and IPSC mean barrages was significantly reduced in \textit{Fmr1-KO} recordings, also introducing an approximately 20ms lag in the genotype mean cross-correlogram peak. Finally, to examine whether a similar shift took place in the relative timing of balanced inhibitory and excitatory charge integration, the cumulative percentage of the total charge transferred by the mean IPSC and EPSC barrages was evaluated for each sample during the 200ms feedback window (Figure 5-8e). In wild-types, the balance of average In/Ex charge integration formed a mixed population, however the majority of neurons integrated \textasciitilde 65\% of their total inhibitory charge by the time that 50\% of the excitatory charge had been integrated. This profile was altered in \textit{Fmr1-KOs}, such that the rate of inhibitory charge integration was slightly slower (approximately 55\% total IPSC charge integration by the time of 50\% EPSC integration on average). This finding is therefore in agreement with the weaker correlation and greater lag in mean In/Ex current envelopes shown in Figure 5-8e.

Overall these data support the conclusion that synaptic input to Layer 4 Ex. neurons during cortical network activity is abnormal in \textit{Fmr1-KOs}, and that disproportionate changes to the amplitude, timing and fidelity of inhibitory input are the predominant effect. These effects are likely contributing factors in the reduced temporal fidelity of Ex. neuron spike times during TC-evoked network sustained activity as demonstrated through cell-

\textsuperscript{54} The outward IPSCs currents were also inverted.
attached recordings in section 5.3.2. As predicted, the finding that inhibitory feedback input lags excitatory network feedback in Fmr1-KOs is consistent with the bidirectionally reduced connectivity between Fmr1-KO FS interneurons and Ex. cells shown by paired recordings in Chapter 3, and with a similar conclusion reached by Gibson and co-workers in a previous (2008) study at older ages, albeit where the change in FS connectivity was unidirectional compared to bidirectional as in the present study. Conceptually, since the FS neurons are more weakly embedded within the Layer 4 network, more Ex. cells would be required to fire before sufficient integration of feedback synaptic charge could take place, driving the FS cell to fire and transmit inhibitory synaptic charge back to Ex. neurons. Weaker synaptic embedding of FS interneurons within the Layer 4 network could also account for the increased trial-to-trial variability in the network feedback inhibition observed in Fmr1-KO recordings.

Interestingly however, bidirectionally stronger release probability of intracortical connections was observed in Chapter 3. Whilst perhaps insufficient to counter the use-dependent rundown in synaptic currents, it is predicted some trade-off through greater fidelity of initial release probability could be achieved in the Fmr1-KOs, although this is likely negligible for the FS-Ex connection as these synapses will be in a depressed state after participation in FFI.

It is harder to predict the behaviour of non-FS interneurons from the current data however. Whilst presented here as total inhibitory synaptic input feedback from the perspective of the voltage-clamped somatic currents of single neurons, a significant remaining unknown is the
contribution of non-FS cells to network feedback inhibition in Fmr1-KOs. Due to their facilitating inputs from local Ex. neurons, and integrating somatic voltage responses, these cells require sustained excitation from local network activity to stimulate their firing (Beierlein et al., 2009). Similar to FS interneurons, intrinsic properties of non-FS interneurons were distorted in Fmr1-KO recordings (see cluster analysis of Fmr1-KO interneurons in Chapter 2), in particular displaying elevated input resistance and longer membrane time constants. Irrespective of potential changes to connectivity that would alter their functional embedding within the Layer 4 recurrent network\(^5\), these effects will slow the integration of excitatory synaptic input by non-FS interneurons, in turn retarding their spiking responsiveness to network activity, and ultimately impact upon the speed at which inhibitory synaptic input is relayed back to Ex. neurons, as demonstrated in this section. Therefore, similar mechanistic if not dynamic disruptions are predicted to affect the two majority pools of NS and non-FS interneurons providing synaptic inhibition in Layer 4, both contributing to the slower feedback inhibition reported here.

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\(^5\) Although not presented in this thesis, a significantly reduced sEPSC rate was recorded in non-FS interneurons from Fmr1-KO slices.
5.3.5. Altered properties of TC-evoked intracortical network activity in Fmr1-KO S1 as resolved by multi-electrode array techniques

5.3.5.1. Choice of recording technique

How do the changes to single-cell current inputs and firing times found by microelectrode recordings in Fmr1-KO neurons affect the dynamic circuit response at the level of the entire Layer 4 network? Such an analysis would likely require the concurrent monitoring of spiking activity from many neurons. Describing the pairwise interactions of neurons could be informative (e.g. Okun and Lampl 2008; Gentet et al. 2010), however, due to the strong inter-cell diversity of intrinsic and synaptic properties among excitatory neurons in the barrel cortex (Lefort et al., 2009; Yassin et al., 2010; Ashby and Isaac, 2011), it was envisioned that a substantial number of paired recordings would be necessary to achieve sufficient analytical power. Additionally, without careful documentation of neuronal position, microelectrode recordings lack necessary spatial resolution to study propagating cortical activity. Techniques such as multi-cell population imaging using Ca\(^{2+}\) indicators can obtain acceptable temporal resolution whilst maintaining excellent spatial resolution. For the present study, considerable effort was expended in optimising the chemical and technical procedure for multi-cell Ca\(^{2+}\) population imaging using synthetic indicators (bulk-loaded Aminomethoxy esters of Oregon Green BAPTA 2 or Fluo-4). Unfortunately at the time of recording, the available epifluorescent imaging equipment produced unacceptable signal-to-noise resolution; newly acquired 2-photon equipment represents an attractive possibility to revisit
this approach. An alternative experimental paradigm that provides meso-scale spatial resolution is Voltage Sensitive Dye (VSD) imaging. This approach has been successfully used to track the spatiotemporal propagation of network activity using a variety of imaging techniques, both in slice preparations and in vivo (Derdikman et al., 2003; Han et al., 2008; Kuhn et al., 2008; Takagaki et al., 2008; Sakata and Harris, 2009; Senseman and Robbins, 2012; Lustig et al., 2013). A potentially serious complication of this approach however is the possibility of unexpected toxicity or pharmacological interaction with neurotransmitter receptors, particularly GABA_A (Mennerick et al. 2010; Grandy et al. 2012, although see Derdikman et al. 2003). Therefore, given the lack of single-cell resolution achievable with VSDs with currently available equipment, and the potential for spurious GABAergic disruption, particularly in light of changes to functional GABAergic properties reported above, this technique was not pursued.

An analogous spatially registered technique was chosen that permits electrical recording over a wide area of tissue, whilst retaining features of single-cell physiology. In this Multi-Electrode Array (MEA) approach (Figure 5-9a), brain slices were positioned on a regularly spaced grid of lithographically printed ITO electrodes, positioned on a glass substrate and sinter coated with Platinum Black.

Exceptionally low impedance is achieved (<22kΩ @5kHz) by this preparation, leading to high quality, high signal-to-noise recordings. Furthermore, a modular system was designed, compatible with standard dual-channel whole-cell brain slice electrophysiology (Ag/AgCl
grounding, fast oxygenated perfusion, heating and vacuum suction etc.), permitting synchronised data acquisition at 20kHz sampling frequency. The current configuration also supports continuous synchronised frame grabbing of reflected epifluorescence via a cooled CCD camera, thus VSD or Ca^{2+} imaging can be obtained during the same experiments. Together, this approach permits a highly detailed and adaptable experimental configuration providing highly nuanced single-cell and network wide physiology across several spatiotemporal scales. In this section, several abnormal features of synaptic and putative spiking activity from multi-unit data\(^56\) are presented from extracellular MEA slice recordings.

**Figure 5-9** Example experimental configuration for Multi-Electrode Array (MEA) recording.

a). The MED64 P515A MUA probe (8x8 channel, 150μm spacing) and amplifier (64 channel, 20KHz sampling rate). b). Example DIC image (4x magnification, Fmr1\(^{+/Y}\), P11) showing positioning of the brain slice on the probe. The area of tissue covering the probe spanned approximately three barrels width and nearly the whole depth (at P11) of the cortical mantle.

ci). Example spatially registered field potentials (fEPSPs) evoked by a single TC stimulus (Fmr1\(^{+/Y}\), P10 animal). Note the reversal in potential sign between the 4\(^{th}\) and 5\(^{th}\) rows of the array, corresponding with the upper boundary of strongly ramified TC innervation. cii). Interpolated Current Source Density (CSD) for fEPSPs shown above averaged over the 10ms following the stimulus artefact. Note the pronounced sink-source reversal corresponding with the change in sign of the fEPSPs above.

\(^56\)Despite considerable technical and signal processing effort, unstable and inadequate isolation of single units was achieved with the current setup (despite good signal-to-noise ratios, the wide, 150 μm inter-electrode spacing was found to be largely incompatible with standard unit isolation techniques typically applied to tetrode data). For this reason putative single unit data is withheld.
5.3.5.2. Recording extracellular activity in S1 cortical slices

**Figure 5-9b** shows a representative example recording using the configuration described above. With an 8x8 grid of electrodes, extracellular potentials spanning the entire depth of the cortical mantle, and a lateral extent of approximately 3-4 barrels could be simultaneously recorded. Consistent with previous multi-electrode findings in young postnatal rodent somatosensory (Molnár et al., 2003; Minlebaev et al., 2011) and visual (Colonnese et al., 2010) cortex, a strong short latency negative field EPSP (fEPSP) was recorded in Layer 4 following TC stimulation (**Figure 5-9ci**) that correlated well with both the visually determined anatomy of Layer 4 under widefield microscopy, and the characteristic physiological properties of Ex. (putatively stellate) neurons in subsequent targeted whole-cell recordings. As found previously using single glass microelectrode mapping (data not shown), electrical stimulation of VB thalamus with intensities typically used in this study evoked fEPSPs in at least two adjacent barrels in the majority of recordings. Consequently, Current Source Density (CSD) analysis of monosynaptic inputs (**Figure 5-9cii**) typically demonstrated a laminar sink/source reversal putatively demarcating the boundary between granular and Infragranular cortical layers.

The power of this experimental approach was used both to examine network activity in Layer 4 and to follow the spatiotemporal evolution of synaptic and spiking responses during intracortically sustained network activity. As before, 5x50Hz stimulation was used to reliably evoke network activity in the majority of trials in brain slices of both genotypes.
**Figure 5-10** provides an overview of the experimental strategy used to analyse extracellular activity: After positioning the slice on the MEA to laterally bracket a barrel with confirmed monosynaptic VB input, burst TC stimulation was used to evoke network activity in the barrel cortex as described above using whole-cell recordings. **Figure 5-10a (left)** shows the peak negative fEPSP potential 100-300ms after the TC stimulation overlaid as a heat map. Raw data were processed in two separate pipelines to isolate simultaneous LFP and MUA on each channel (**Figure 5-10a, right**). In a subset of recordings, single cells or pairs of neurons in the Layer 4 barrel showing the strongest amplitude network-sustained fEPSP response\(^{58}\) (**Figure 5-10b**) were targeted for whole-cell recordings. In these recordings, trial-to-trial variability was observed in both the amplitude and the timing of network activity, but these were typically well correlated between LFP and MUA on the same channel (shown expanded in **Figure 5-11**), and the firing and subthreshold responses in whole-cell recordings from neurons directly (i.e. 400\(\mu\)m) above the recording electrode (**Figure 5-10c**). Furthermore, the timing of the LFP/MUA response envelope was found to be broadly consistent with that of single cell responses reported in Layer 4 above.

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\(^{58}\) This barrel did not always receive the strongest monosynaptic fEPSP however.
Figure 5-10 Time-course of TC-evoked intracortical network activity as recorded by extracellular MEA.

a). Left: Example recording (Fmrt<sup>+/−</sup>, P11) showing overlaid the maximum (negative) field potential deflection between 100-200ms after TC stimulation at 5x50Hz. A Layer 4 Ex. (putative spiny stellate) neuron in the barrel showing the greatest fEPSP amplitude deflection was subsequently targeted for whole-cell recording (blue). Right: Signal-processing strategy to separate local file potential (LFP) and multi-unit activity (MUA) from the raw extracellular potential recorded from the MEA channels.

b). Ten overlaid trials (0.07Hz inter-trial interval) showing LFP responses from the example recording shown above. The channel highlighted in green shows the strongest mean LFP deflection. Full scale for each channel: 1s/200μV.

c). Five expanded trials from b), highlighting the time-course of LFP, MUA for the strongest responding Layer 4 channel and a simultaneous whole-cell recording from a Layer 4 Ex. neuron directly above he MEA electrode.
### Network activity on strongest channel in L4

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>LFP</th>
<th>MUA</th>
<th>Wh/Cell</th>
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<tbody>
<tr>
<td>1</td>
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<td><img src="image" alt="MUA Waveform" /></td>
<td><img src="image" alt="Wh/Cell Waveform" /></td>
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<tr>
<td>3</td>
<td><img src="image" alt="LFP Waveform" /></td>
<td><img src="image" alt="MUA Waveform" /></td>
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<td>4</td>
<td><img src="image" alt="LFP Waveform" /></td>
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<td>5</td>
<td><img src="image" alt="LFP Waveform" /></td>
<td><img src="image" alt="MUA Waveform" /></td>
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<tr>
<td>...</td>
<td><img src="image" alt="LFP Waveform" /></td>
<td><img src="image" alt="MUA Waveform" /></td>
<td><img src="image" alt="Wh/Cell Waveform" /></td>
</tr>
</tbody>
</table>

- **LFP (0.1-300Hz filter)**
- **MUA (0.3-5KHz filter)**

**Peak response Amplitude**

- -450 µV
- -100 µV

**Raw data**

**Peak response Amplitude**

- 500 µm
- 500 µm
Figure 5-11 Trial-to-trial variability in the propagation of TC-evoked intracortical network activity.

Ten trials overlaid showing LFP (a) and MUA (b) for example recording in Figure 5-10. Each trial is shown as a different colour, colours are consistent between a) and b).
Local Field Potential (LFP)

5x50Hz stimulation, ten trials overlaid

Multi-Unit Activity (MUA)
5.3.5.3. Altered timing of LFP activity in Fmr1-KO Layer 4

The response properties of TC-evoked sustained LFP activity were first compared between wild-type and Fmr1-KO slices. Figure 5-12a further expands the example wild-type recording show in Figures 5-10,11, highlighting the trial-to-trial variability for polysynaptic network activity recorded on the Layer 4 channel that showed the strongest negative amplitude deflection (shown on top right as a heat-map – note that for this recording the activity started at the extreme left hand side of the array before spreading right). The channel that was most consistently (i.e. modal) the strongest responding channel was chosen, rather than taking values from the strongest responding channels on a trial-to-trial basis. Analysed in this manner, Figure 5-12b plots the mean LFP recorded on the modal strongest responding channel, averaged for 15 wild-type and 9 Fmr1-KO animals. Trial-to trial variability in the position of the Layer 4 channel that showed the greatest LFP amplitude was not significantly different between genotypes. Whilst the absolute amplitude (or the trial-to trial variability) of the peak LFP deflection was not significantly different between genotypes (Figure 5-12c), Fmr1-KO LFP minima peaked significantly earlier compared to those from wild-types (Figure 5-12d).

Trial-to trial variability in the LFP peak latency was not significantly different between genotypes. Interestingly, the mean wild-type response showed a slight shoulder effect that distinguished the last decaying direct TC fEPSP from a subsequent delayed LFP component that then continued to build before peaking ~60ms after the last stimulus (Figure 5-12b, left). This was not observed in the Fmr1-KO mean response, such that the two
components appeared fused. One interpretation of this data that will be explored below is that greater excitation is achieved by thalamocortical input in $Fmr1$-KOs, as shown through single-cell recordings in Chapter 3, but that network activity sustained by intracortical firing is comparatively weaker.

5.3.5.4. Lower density of multi-unit activity in $Fmr1$-KO Layer 4 during network-sustained firing activity

Consistent with this interpretation, when the rate of multi-unit activity (MUA) was examined on the same (strongest Layer 4) channel for that LFP was analysed on for recordings presented above, strong changes were observed between genotypes. As with single-cell data, the delayed 200ms window for feedback activity was again imposed on the analysis of network-sustained MUA to reduce contamination from spiking from direct TC-evoked spikes (Figure 5-13a). In wild-type slices, the mean MUA density increased transiently during this window to peak at approximately 150ms after the first TC stimulus, before declining slowly during the subsequent 150ms (Figure 5-13b). In $Fmr1$-KO recordings however, MUA density was slightly lower at the start of the window and rather that increasing with time, steadily decreased during the window, such that between 125-205ms the mean MUA density was significantly reduced in mutant recordings compared to in slices from littermates. This demonstrates that the firing rate of TC-evoked network activity in Layer 4, as observed from the summed extracellular AP waveforms of multiple putative spiking neurons, is significantly reduced in slices from P10-11 $Fmr1$-KO animals.
Figure 5-12 Timing and amplitude of polysynaptic fEPSPs during TC-evoked cortical network activity.

a). Left: Ten overlaid trials expanded for the strongest responding channel in Figures 5-10,11. Right: heat maps showing amplitude (top) and latency (bottom) for the MEA recording on the left (average of ten trials) between 100-300ms post stimulus. 

b). Left: Grand mean±SEM fEPSPs on the strongest responding Layer 4 channels for recordings from 15 Fmr1+/y and 9 Fmr1−/Y animals. Right: The trial-to-trial variability in the position of the strongest responding channel was not significantly different between genotypes (p>0.05, Mann-Whitney). 

c). The maximum negative fEPSP amplitude on the strongest channel between 100-300ms, or the trial-to-trial variability were not significantly different between genotypes (p>0.05, Mann-Whitney). 

d). Latency to fEPSP peak amplitude was significantly faster in Fmr1−/Y recordings compared to wild-type littermates (134±5.8 vs. 108±6.3, p=0.01, Mann-Whitney). Trial-to-trial variability was not significantly different.
Strongest Layer 4 channel (10 trials overlaid)

Position of strongest channel (trial-to-trial stability)

Peak Latency (ms)

Peak Latency variability (SEM, ms)
Figure 5-13 Altered rate of multi-unit activity on the strongest Layer 4 channel in Fmr1<sup>−/−</sup> recordings.

**a).** Example MUA from the P11 Fmr1<sup>+/−</sup> recording shown in Figure 5-10 to -12 during a window 100-300ms post-stimulus window. Green shaded region is the channel that showed the strongest LFP deflection during this time period. MUA rate was quantified by half-wave rectifying high-frequency filtered extracellular waveforms, followed by normalizing to R.M.S. baseline noise amplitude and thresholding at 8x r.m.s noise. Detected threshold-crossing peaks were then convolved with a Gaussian PDF with sigma=5ms. MUA in this figure is for a single trial, while rate estimates (black smooth lines) are mean across 10 trials.

**b).** Mean±SEM MUA density for the strongest Layer 4 channel in recordings from 15 Fmr1<sup>−/−</sup> (blue) and 9 Fmr1<sup>+/−</sup> (red) animals. Average MUA rate was lower in recordings from mutant mice during this period compared to in wild-type recordings. Mean rate compared between genotypes in 5ms bins was significantly reduced for the period between 125ms and 205ms, and overall peak MUA density between 100-300ms was significantly reduced in Fmr1<sup>−/−</sup> recordings (p<0.05, Mann-Whitney).
a  MUA on strongest L4 channel

b  

MUA density $p$(spike/5ms) vs. Post-stimulus time (ms)
5.3.5.5. Abnormal spatiotemporal propagation of synaptic activity in *Fmr1-KO* barrel cortex

To extend the previous analyses, the precise spatial registration of cortical field potentials provided by the regularly spaced MEA electrodes was used to study the spatiotemporal propagation of TC-evoked intracortical activity.

**Figure 5-14** presents the analysis strategy that was adopted for this approach. The array-wide LFP signal arose from distributed intracortical network activity. For a representative wild-type recording, in **Figure 5-14a** this is displayed as a 3D interpolated voltage map every 50ms during the 200ms post-stimulus window of analysis. With this display scheme, negative deflections (hyperpolarisation of the extracellular space, largely corresponding to net neuronal excitation; see Buzsáki et al. 2012 for review) are represented as blue valleys, whilst depolarisation of the extracellular space are shown as red. Following an initiation that was always observed on electrodes putatively beneath Layer 4, negative LFP deflections propagated across the cortex in a pulsatile manner reminiscent of a travelling wave-front, subsequently followed by prolonged positive LFP deflections.

Since the spatial extent of LFP minima typically spanned an area >>1 electrode in these recordings, and can in general be detected up to several mm away from the source of synaptic activity in conductive tissue (Buzsáki et al., 2012), Current Source Density analysis (CSD – see methods) was

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60 This is presented for visual clarity, however, un-interpolated data at 50 μs (i.e. native 20kHz) sampling intervals were used for subsequent analyses.
employed to improve spatial localisation of the focus of electrical excitation at each time-point. CSD decomposition revealed the spatial progression of a tightly focussed current sink, followed by a more diffuse current source that outlasted the sink by several hundred milliseconds. Taking the position of the current sink on the underlying MEA channel as the focus of network at each time-point, the movement of the network activity between channels was evaluated (Figure 5-14b,c). Importantly, the distance moved away from the site of initiation was analysed and would be hard to obtain at the same resolution using any other electrophysiological technique. Finally, the speed of sink propagation was calculated from the average displacement per time-step.
Figure 5-14 Analysis strategy for evaluating intracortical network activity propagation. Single trial example is shown.

a). Five snapshots at 50ms increments from interpolated maps of cortical activity, showing Local Field Potential (LFP, top) and corresponding Current Source Density (CSD, middle). For comparison, Multi-Unit Activity (MUA) is shown below. Activity plots show a single trial example polysynaptic network activity sustained by intracortical firing for the example recording (Fmr1+/Y, P11) shown in Figures 5-10 to 13. In this example, the TC-evoked activity was initiated in a barrel at the lateral edge of the electrode array, before growing in lateral extent and propagating in a medial direction. Note that troughs/valleys in the LFP and CSD plots correspond to neuronal depolarisation as observed from the extracellular space, and that the position of the focus of activity (two-dimensional minima) is better defined in the CSD compared to the LFP recording, at the cost of more noise for small amplitude, local voltage deflections. Also note that spiking activity as resolved by the MUA, propagates to deeper cortical layers in this example.

b). Spatial position of the peak CSD signal: Movement of the CSD signal was used to track the position of the strongest current sink at each time step during the 200ms window of analysis. Fraction of time during which this activity occupied each channel is shown as a heat map here. Actual trajectory is hidden for clarity.

c). The Pythagorean vector of sink movement between successive time-steps for the recording shown in a). is summarised as a circular histogram with 45° bins.
These analyses were applied to MEA recordings from 15 wild-type and 9 Fmr1-KO animals (same data as Figures 5-12,13). Firstly, no significant difference in the speed of sink propagation was observed between genotypes (Figure 5-15a, right). For both genotypes, the direction of sink propagation was typically in a supragranular direction, often with lateral movement across upper cortical layers. The distance propagated by the sink centre was compared between genotypes as a function of time in the 200ms window of analysis (Figure 5-15b, top). Wild-type recordings showed moderate acceleration during the first ~10ms followed by slower, consistent movement away from the site of origin during the next ~190ms. However in Fmr1-KO slices, the period of rapid movement appeared to last slightly longer, such that between 100-200ms the current sink was on average further from the site of initiation in Fmr1-KO recordings compared to in wild-types recordings. Subsequently, Fmr1-KO sinks appeared to stop moving; a plateau in distance propagated was observed from approximately 200ms onwards. Correspondingly, a significant difference in mean sink propagation distance was observed between genotypes during the last 20ms.

During the same time period, the number of MEA channels covered by the sink activity was not constant. Initially, in wild-type recordings, seven surrounding channels on average showed sink amplitudes within 60% of the peak sink density at the centre of the burst (Figure 5-15b, bottom). Correspondingly, an area of co-active cortical tissue ~875μm² was typically sustained during the first ~50ms, before gradually reducing to that covering a single channel (~125μm²) over the following 150ms. Strikingly,
in Fmr1-KOs, whilst on average cortical tissue above 3 channels (~375μm²) contributed to sink activity at the start of the window of analysis, this was not sustained contracted almost immediately to <2 channels, such that a significantly reduced area of cortical tissue on average contributed to sink activity from 120ms onwards.
Figure 5-15 Altered spread of network activity in Fmr1-/-Y recordings. Data in this figure represent the mean of 10 trials from 15 Fmr1+/-Y and 9 Fmr1-/-Y animals.

a). Left: Schematic representation of the analysis approach, showing the position (at a point in time) of the strongest current sink (green), and the number of channels (yellow) with sink amplitude greater than 80% of the strongest centre channel. Right: Average sink propagation speed away from position at t=100ms was not significantly different between genotypes: grand average histogram of propagation speed from ten trials from each recording, binned for the whole 200ms recording (p>0.05, Mann-Whitney). Green and black arrows schematise movement of the strongest and coactive surround current sink, respectively.

b). Top: Absolute distance traversed by strongest cortical current sink during 200ms of recording. Mean of 10 trials for each recording. Data show mean±SEM for each time-point at 20kHz sampling rate. Recordings from mutant slices plateaued during the last 20ms, while wild-type recordings continued to propagate: Mean distances from starting position at t=100ms were significantly different between 280-300ms (p<0.05, Mann-Whitney, comparing data downsampled to 5ms resolution).

Bottom: At each time-point, the area of tissue (calculated from the number of channels) with >80% current sink amplitude compared the strongest channel at that time. Data shows mean±SEM for each genotype. A significantly smaller area of tissue was simultaneously active in Fmr1-/-Y recordings compared to those from Fmr1+/-Y recordings from 120-300ms (p<0.05 Mann-Whitney, comparing data downsampled to 5ms resolution). Similar significant results were obtained using raw LFP data, or by relaxing threshold to 60% of peak amplitude (data not shown). Note that this finding is further supported by the weaker propagation of intracortical multi-unit rate between channels in Fmr1-/-Y recordings (shown in subsequent figures).
a

Co-active surround
Strongest sink

b

Distance of
cortical current sink
from starting position (µm)

Simultaneously co-active cortical
Area (mm²)

Fraction of time (%) vs. Sink propagation speed (m/s)

Fraction of time (%) vs. Post-stimulus time (ms)
5.3.5.6. Restricted spatiotemporal evolution of spiking activity in Fmr1-KO Layer 4

In addition to the attenuated spatial propagation of synaptic activity presented in the previous section, spatial evolution of spiking activity sustained by TC-evoked network activity was found to be weaker in Fmr1-KOs during the same time window for analysis. Examples showing the spatial propagation of MUA activity during network-sustained firing in wild-type and Fmr1-KO recordings are presented in Figure 5-16a.

Figure 5-16 Intracortical network spiking activity evoked by TC burst stimulation is spatially and temporally confined in Fmr1-KOs and follows a more stereotyped pattern

a). Multi-Unit activity density for example recordings from Fmr1+/Y (top) and Fmr1−/Y (bottom) slices. MEA channels are ordered from top to bottom by first MUA onset time. Note the lower density, shorter duration and less spatially complex trajectory in the Fmr1−/Y example.

b). TC-evoked intracortical spiking activity was less dimensionally complex in Fmr1−/Y recordings: Mean Principle component (PC) decomposition of MUA signals between 100-300ms (bars) and cumulative variance explained (lines). The top 3 PCs explained more variance in Fmr1−/Y MUA signals compared to those from wild-types.

c). Projection of the top 3 dimensions of Fmr1+/Y (15 animals) and Fmr1−/Y (9 animals) MUA signals between 100-300ms post stimulus into the same principle component space. Each line shows the mean trajectory of ten repeated trials, the width of each line corresponds to the average distance between trajectories for individual trials and the mean response trajectory for each time-point.

d). Average distance between MUA trajectories (calculated for top 3 PC’s) for individual trials and the mean response trajectory at each time-point (left) and cumulatively for during 200ms (right). Data shown are mean±SEM for Fmr1+/Y (blue, 15 animals) and Fmr1−/Y (red, 9 animals). Peak and total cumulative trial-trial trajectory variability were significantly reduced for Fmr1−/Y trajectories (Mann-Whitney, p=0.004 and p=0.014, respectively).
Figure a shows a heatmap of MUA trajectories for Fmr1+/Y (n=15) and Fmr1-/-Y (n=9) mice. The x-axis represents post-stimulus time (ms), and the y-axis shows channel number. The color scale indicates the probability of a spike (p(spike)).

Figure b illustrates the variance explained by the principle components. The graph compares Fmr1+/Y (n=15) and Fmr1-/-Y (n=9) mice, showing how much variance each principle component captures.

Figure c features MUA trajectories, averaged over ten trials, with a visualization of trial-to-trial variability.

Figure d displays the trial-to-trial variability and cumulative variability, with a marked * indicating significant differences.
In the wild-type example, MUA density increased rapidly on the initiating channel, and rapidly propagated to the majority of channels on the MEA, with approximately half of the electrodes simultaneously detecting high rates of MUA firing at the peak of network activity. Conversely in the Fmr1-KO example recording, in addition to displaying substantially reduced MUA density across all channels (as quantified on the initiating Layer 4 channel in Figure 5-13), MUA showed markedly reduced spread onto neighbouring channels. In addition to the large extent of spatial spread observed in wild-type recordings, variability in MUA propagation was high both between brain slices and for repeated trials (as has been noted in Layer 4 during sensory processing in vivo: Haslinger et al. 2006).

Therefore, dimension reduction was applied before comparing inter-trial and inter-animal variability. This technique has previously been applied successfully to the description of spatially complex VSD imaging data (Derdikman et al., 2003; Olivas et al., 2012; Senseman and Robbins, 2012), as well as more extensively in describing neuronal population dynamics (Brown et al., 2005b; Einevoll et al., 2009; Raman and Stopfer, 2010; Assisi et al., 2012; Churchland et al., 2012). Principle component analysis was applied to the MUA dataset: for each channel, MUA density across recorded in ten repeated trials and the resulting 2D vector was concatenated (i.e. (200ms x 10 repeats) x 64 channels), normalised by z-score and decomposed into principle components. Mean results for the PCA decomposition of MUA in the current P10-11 dataset are shown in Figure 5-16b. As expected for a dataset with reduced spatial spread, the top three principle components explained ~8% more variance on average for
*Fmr1-KO* recordings, largely dominated by a greater contribution from the 1st component. When projected into a common principle component space (*Figure* 5-16c), propagation of multichannel MUA recordings were observed as looping, parabolic burst trajectories.

*Fmr1-KO* trajectories were notably compressed on average, with the majority of activity restricted to the first two dimensions. This suggests that the extent of TC-evoked MUA firing generated and sustained by network activity is less complex overall in *Fmr1-KOs*: both spatially smaller and lower in rate. Furthermore, the variability in MUA trajectories between trials was compared between genotypes by calculating the Cartesian distance between each individual trial’s trajectory and the geometric mean trajectory (for the top 3 PC’s) at each time-point - summarised in *Figure* 5-16d. Significant inter-trial variability was able to accumulate during 200ms of network sustained firing in wild-type slices, with the maximum inter-trial diversity occurring between 100-200ms post-stimulus. In *Fmr1-KO* recordings however, the accumulation of variability was significantly depressed, and the average inter-trial diversity was significantly lower, although showing the same early peak and early (100~120ms) diversity.

Taken together with the analysis of cortical synaptic activity propagation shown by current sink tracking in *Figure* 5-15, these MUA data demonstrate that spatiotemporal evolution of putative spiking activity is weaker and more stereotyped in *Fmr1-KO* recordings.
5.3.5.7. *Fmr1-KO* Layer 4 network-sustained LFP demonstrates elevated gamma-band but reduced high-frequency spectral power

What mechanism underlies the weaker propagation of network activity in *Fmr1-KOs* shown in MEA recordings in the previous sections? The depressed spiking rate is consistent with an increased feedback inhibitory relative to excitatory synaptic input, as shown by whole-cell recordings in Section 5.3.3.1, while the spatially tighter current sinks could represent the effect of stronger centre-surround inhibition.

Data presented in Chapter 3 showed changes to connectivity between neighbouring excitatory and inhibitory FS neurons, and a reduction in spontaneous EPSC rate was also observed in *Fmr1-KO* Layer 4 Ex neurons. It is impossible to rule out an effect of reduced presynaptic release probability from analysis of spontaneous currents alone, however this is unlikely at least at TC synapses since enhanced STD was observed in Chapter 4). It is thus likely that this effect is dominated by reduced recurrent excitatory connectivity within Layer 4. Overall, the elevated feedback inhibition observed in Section 5.3.3.1 suggests that disruptions to functional excitatory circuitry in *Fmr1-KOs* could be more severe than those to inhibitory circuitry at this age. This forms the subject of ongoing experimentation and simulation.

Previous studies on processing in recurrent cortical networks suggest that distinct frequency components of the extracellular LFP can be dominated by the coherent firing of different cell types during network activity. For example, Cardin and colleagues (2009) showed that by selectively driving
FS interneurons in mouse V1 using optogenetic techniques, the power of gamma (30-80Hz) activity in LFP could be strongly enhanced with little effect on gamma centre frequency when stimulation intensity was varied. Conversely, selectively driving excitatory neurons produced diffuse low-frequency LFP activity. From this data, the authors concluded that cortical gamma oscillations are a resonant circuit property and went on to show that the precise phase of artificially induced gamma oscillations profoundly influence the timing of sensory-evoked spikes in excitatory neurons. This study is supported by previous work that suggest that feedback excitatory input to a resonating inhibitory gamma oscillatory system provides a predominantly stabilising effect (Whittington et al., 1997; Börgers et al., 2005).

One prediction stemming from these findings therefore, is that if FS interneurons are more active during circuit activity in a population of cells than neighbouring Ex. neurons, or, if their transmitted synaptic currents are more coherent in postsynaptic Ex. neurons than those of recurrent excitatory inputs, then this should translate to a higher relative power in gamma frequencies of LFP. To test this hypothesis on the current P10-11 Fmr1-KO dataset, Fourier spectral decomposition of LFP recorded during feedback circuit activity was applied to the strongest MEA channel under Layer 4 (Figure 5-17a). To maximise the spectral-temporal resolution of spectra, multi-taper windowing was applied to data before construction of spectrograms (using routines from the Chronux toolbox: Bokil et al. 2010). Figure 5-17b shows spectrograms constructed from example wild-type and Fmr1-KO LFP recordings.
Figure 5-17 Frequency decomposition of layer 4 LFP signals during TC-evoked intracortical network activity: Example spectra for Fmr1-KO and wild-type recordings.

a). Single trials TC-evoked LFP network responses on the strongest responding Layer 4 channels for example recordings. Dashed lines show the mean of 10 trials for reference.

b). Multi-taper Fourier spectrograms (10Hz time-bandwidth product, 5 tapers) calculated from single-trial examples shown above, with spectral power density expressed normalised to mean power during 100ms baseline period (-100 to 0ms). Black vertical lines indicate the boundaries of the 200ms window used for feedback activity analysis. Red vertical lines indicate spectral slices at the time of LFP minima during this window.

c). Black: Mean±SEM power spectra averaged during the 200ms window. Red: spectra aligned to the time of LFP minima (±2.5ms).
Layer 4 LFP

(a) $Fmr1^{+/\gamma}$

(b) Frequency (Hz)

(c) $dB (\mu V^2/Hz)$

Frequency (Hz)
For both recordings, the power spectra were dominated by low frequency activity corresponding with slow fluctuations in the LFP. In the wild-type case however, a moderate power increase at 50Hz was observed during TC-evoked network activity, but notably a strong contribution to power at ~90Hz was observed aligned to the LFP minimum, in good temporal correlation with fast oscillatory activity observed at this time. This was unlikely to be a harmonic of lower gamma activity since its time-course did not perfectly match that of the gamma peak and its central frequency was not an exact integer multiple of the lower gamma activity. In the Fmr1-KO example however, the gamma oscillatory activity represented a larger contribution of total spectral energy, and a diminished contribution of higher (~100Hz) power was observed. Correspondingly, this was reflected by the mean LFP power spectra between 100-300ms (shown in black), and the power in the 5ms bin aligned to the LFP peak (shown in red) in Figure 5-17c. The spectra varied between trials with the timing and oscillatory pattern of the LFP. Moreover, the peak gamma frequency was variable between trials, as shown by green circles in Figure 5-18a.

To quantify the network-sustained gamma activity between genotypes, mean power spectra across 10 trials was calculated for 200ms after the stimulus onset. Mean power spectra are shown in Figure 5-18b. As predicted, a significant increase in the power between 30-80Hz was observed in Fmr1-KO recordings, and more precisely, the spectral power at the peak gamma frequency for individual recordings was significantly elevated. Interestingly, the mean frequency at which the gamma peak occurred was not significantly different between genotypes, although it is
Figure 5-18 Elevated gamma spectral power in Fmr1-KO recordings during sustained intracortical LFP activity evoked by TC burst stimulation

a). Example multi-taper spectrograms, averaged between 100-300ms post-stimulus for each MEA channel, with the spectrum for each trial calculated individually. Right: Expanded spectra for ten repeated trials on the strongest Layer 4 channel. Green markers indicate local maxima detected between 30-80Hz corresponding to the peak power at “gamma-band” frequencies.

b). Left: Mean±SEM power spectra for Layer 4 LFP responses from Fmr1+/− (15 animals) and Fmr1−/− (9 animals) recordings, averaged across 200ms for each of 10 repeated trials successfully evoking polysynaptic network activity. Asterisk indicates a significant increase in the average power between 30-80Hz in Fmr1−/− recordings (p<0.05, Mann-Whitney). Right: Gamma-band centre frequency was not significantly different between genotypes, but the power at this frequency was significantly elevated in mutant recordings (p<0.01, Mann-Whitney).
noted that this spectral peak was broader in *Fmr1-KOs* compared to wild-type recordings (compare spectral spread of gamma lobes of individual examples in Figure 5-17b and mean spectra in Figure 5-18b). Furthermore, a strong trend towards increased inter-trial variability was observed in the peak gamma frequency in *Fmr1-KO* recordings, suggesting that the timing of gamma activity was unstable between repeated circuit activations. Due to variable timing and duration of TC-evoked network activity, this is likely to represent an imprecise estimate of spectral power. By binning data for 200ms of fast oscillatory activity (potentially up to ~10 cycles of gamma oscillations at 50Hz), the fine precision of oscillatory activity is lost. Furthermore, whilst Fourier and Hilbert-based spectral decomposition techniques typically decompose signals into functions that are well localised in frequency, they are temporally imprecise (typically smeared ~100ms). This becomes a significant impediment to quantifying short bursts of high frequency activity, typical of high gamma-band activity, or the summed potentials of coherent AP waveforms. These arise as sharp transients, but cannot be represented by these methods using a single function, instead appearing in spectrograms as nested oscillations across multiple frequencies. Therefore, to minimise the effect of temporal averaging on the analysis of high frequency components associated with spiking activity, a different frequency decomposition approach was taken that permitted higher spectral-temporal resolution (Ray and Maunsell, 2010). 300ms of layer 4 LFP signal was excised during TC-evoked network activity and aligned by the LFP minima for each trial. Matching Pursuit (MP) frequency decomposition was then applied to each trial individually.
Figure 5-19 TC-evoked network activity in Fmr1-KO Layer 4 shows reduced power in high frequency bands associated with spiking waveforms at the time of peak LFP amplitude.

a). Mean matching pursuit spectrograms for Layer 4 LFP network activity with signals time-shifted to align LFP minima, showing 100ms/200ms before/after LFP peak. Data are mean of 10 trials for Fmr1+/Y (15 animals) and Fmr1−/Y (9 animals) MEA recordings. Overlaid (white) are the time-shifted LFP signals for each genotype (mean±SEM, averaged over 10 trials for each animal). Note the less prominent sharp LFP minima for the average Fmr1−/Y signal.

b). Mean±SEM spectral power as calculated in the above analysis, at the time of the LFP peak. A significant reduction in mean spectral power between 90-120Hz was observed in Fmr1+/Y recordings in Layer 4 (p<0.05, Mann-Whitney).
MP is analogous, but considered superior to wavelet decomposition for the study of fast oscillatory components (Mallat and Zhang, 1993; Ray et al., 2008; Bénar et al., 2009). Briefly, in this approach, an overcomplete dictionary of time-frequency elements is constructed, including “Gabor atoms” – Gaussian convolved sinusoids, as well as pure Fourier sines and Dirac $\delta$ functions (to provide perfect sinusoids and impulses, respectively). These are then iteratively subtracted from the input signal using a two-step error function until the residual has no further explainable activity. MP was performed on temporally aligned LFP for each individual trial of TC-evoked network activity with 0.5ms and ~0.5Hz temporal and spectral resolution, respectively, with spectral power expressed as a logarithmic ratio (in dB) of that in an equal duration of oscillatory activity at baseline (Figure 5-19a). In the mean wild-type MP spectrum, strong transient spectral energy was observed between 80~130Hz, and ~180Hz aligned with the peak of the average LFP waveform. This formed a sharp minimum, therefore these spectral components are likely to reflect bleedthrough of slower components of summed, correlated, extracellular spike waveforms to the LFP signal (Ray et al., 2008). The mean Fmr1-KO LFP peak lacked the prominent sharp minima observed in the wild-type mean response. Correspondingly, the high frequency spectral components lacked the same energy of the wild-type. MP spectra with 0.5ms precision at the peak of the LFP (t=100ms) are summarised in Figure 5-19b. Fmr1-KOs showed a significant reduction in spectral power averaged between 90-120Hz, compared to wild-types.
Together, these data on spectral content of Layer 4 LFP during network-sustained firing activity are consistent with both elevated gamma (30~80Hz) and reduced high frequency (~100Hz) components. In light of the data in the single cell data presented in this chapter showing stronger but temporally imprecise feedback inhibition and imprecise spike times, and lower MUA firing on Layer 4 channels in Fmr1-KOs, this is interpreted as a population level report of dominant rhythmic GABAergic firing in combination with a reduction in the rate and correlation of spiking activity.

5.3.5.8. Spike-LFP, and LFP-LFP interactions across long distances

To what extent are these changes on a single electrode under Layer 4 (~125 μm²) representative of network synchrony across a wider area? Spectral coherence between LFP on the most active Layer 4 centre channel and surrounding, co-active channels was investigated for frequencies between 1~200Hz during the 200ms window for feedback activity analysis (Figure 5-20a).

Unfortunately, the methodology used in estimating coherence (multi-taper Fourier spectra) averaged across the whole 200ms period, and as discussed above, the LFP propagated in a wavefront-like manner with brief periods of oscillatory activity observed at the centre channel. Therefore, this analysis lacks the necessary temporal precision to analyse this effect – high levels of coherence were observed in all cases and little difference was resolved between individual trials, recordings, or genotypes.
Figure 5-20 Spectral coherence and phase as a function of frequency components observed during TC-evoked intracortical network activity (i.e. 100-300ms post-stimulus). Data shown are mean±SEM of recordings from 15 Fmr1+/Y animals and 9 Fmr1−/Y animals, mean of 10 trials in which network activity was successfully recruited.

a). Average coherence between LFP on strongest Layer 4 channel and LFP on surrounding co-active channels (Time-Bandwidth product = 3, 5 tapers. b). Average within channel coherence between LFP and MUA, averaged across co-active channels (Time-Bandwidth product = 20, 4 tapers. c). As in b), but restricted to the strongest Layer 4 channel.
**LFP-LFP coherence**
(strongest Layer 4 channel vs. coactive channels)

**MUA-LFP coherence**
(mean across coactive channels)

**MUA-LFP coherence**
(on strongest Layer 4 channel)
A more sensitive approach could possibly involve multi-taper or wavelet “coherograms”, time-locked to the propagating wavefront. Similarly spuriously flat coherence spectra were obtained for MUA-LFP spectra, both averaged between simultaneously co-active channels (Figure 5-20b) and restricted to coherence on the strongest Layer 4 channel only (Figure 5-20c). For these analyses, single-trial Gaussian-convolved MUA density functions were used, and it is noted that results obtained were highly sensitive to the binning of MUA counts. This data is considered inconclusive at the present time. An interesting shift in the frequency dependence of LFP-MUA phase of coherence was observed however (Figure 5-20b, right). Spike-triggered average LFP analysis would likely be informative in further disentangling this interaction (discussed below). In the absence of sufficient single-cell or single-unit data, quantification of this preliminary data is not reported in the current study. Ideally, to directly examine these interactions, spike times of known cell types would be registered against the LFP, and spike-triggered averaging would be used to probe spike-LFP phase interactions. Since imperfect single-unit isolation has been obtained with currently used analysis techniques, this has not been possible. The MUA offers a surrogate dataset but is temporally imprecise and lacks information about cell type. Efforts to study spike-field interaction using targeted single and paired whole-cell recordings are ongoing – an example paired recording from two Layer 4 neurons with simultaneous filtered gamma (30-80Hz bandpass) activity on the nearest Layer 4 MEA channel is shown in Figure 5-21. It will
be particularly interesting to quantify both cell-to-cell firing correlation, and the stability of phase-preference between multiple trials.

Figure 5-21 Example paired recording (wild-type, P11) from two adjacent Layer 4 Ex. neurons showing simultaneous gamma-band filtered oscillatory activity (green) evoked by TC burst stimulation at 50Hz (orange arrows).

Whole-cell traces are scaled by the same factor for clarity, APs are not truncated. Note the inter-cell and inter-trial diversity in firing responses relative to oscillatory gamma activity in these recordings.
5.3.5.9. Reduced cortical centre-surround MUA correlation in \textit{Fmr1-KO} recordings

In the absence of sufficient single-unit data, spiking correlation was comparing across large areas of tissue by comparing the MUA signal across multiple MEA channels.

However, a confounding factor in the analysis of MUA from thresholded rate estimates as presented up to this point, is that a substantial fraction of potential spiking activity is by necessity discarded to preserve signal-to-noise ratio. In the analyses presented above, a relatively harsh threshold for MUA detection (8x R.M.S. noise at baseline) was employed. Thus, it is possible that only high amplitude spike waveforms (i.e. cells close to the recording electrodes), and/or highly synchronous spiking activity are detected through this approach. In order to compare the synchrony of MUA between simultaneously active channels imposing fewer detection artefacts, the mean cross-correlation between the strongest Layer 4 channel and the surrounding co-active channels was calculated from the raw MUA (i.e. high-pass filtered extracellular waveforms, without thresholding). This analysis is shown in \textbf{Figure 5-22}. Full one-second waveforms were used, including the pre-stimulus, peri-stimulus and network-sustained periods (see \textbf{Figure 5-13a} for example raw data).
**Figure 5-22** Reduced cross-correlation between multi-unit activity on the strongest Layer 4 channel and surrounding, co-active channels during Fmr1-KO TC-evoked cortical network activity

**a.** Mean time windowed cross-correlograms between high-pass filtered extracellular waveforms on the Layer 4 centre channel vs. the surrounding co-active cortical channels. The Layer 4 centre channels were defined by network activity generated current sink amplitude relative as in Figure 5-15b. Note that z-scored MUA data, not MUA density functions are used for this analysis. Data show mean of recordings from 15 Fmr1^{+/Y} animals and 9 Fmr1^{-/-Y} animals, mean of 10 trials in which network activity was successfully recruited. Dotted line indicates zero-lag correlation.

**b.** Orthogonal view along dotted lines in a) comparing zero-lag correlation between genotypes. MUA correlation between co-active channels was significantly reduced during network-sustained firing in Fmr1^{-/-Y} recordings compared to those from littermates. Asterisk denotes significantly reduced mean centre-surround MUA cross-correlation for times between 120-300ms post-stimulus (p<0.05, Mann-Whitney).
Before analysis, stimulus artefacts were blanks and each pair of traces were normalised by z-score. Cross-correlation between pairs of waveforms was then calculated in 5ms moving windows, smoothed by 1ms overlap between successive windows. Population mean centre-surround correlograms are shown in Figure 5-22a, where red and blue regions represent positive correlation and anti-correlation between waveforms, respectively. When the mean zero-lag correlation was compared between genotypes (Figure 5-22b), a striking significant reduction in the mean correlation coefficient was observed in Fmr1-KO recordings during network-sustained MUA between 120-300ms after TC stimulation.

It is unlikely that this finding is an artefact of differential spiking rates between genotypes. Firstly, raw extracellular waveforms were used, and were normalised to provide similar amplitudes before analysis. Secondly, both lower MUA rates, and fewer co-active channels were found in Fmr1-KOs. This would be expected to increase the mean inter-channel correlation. Finally, reduced trial-trial variability in the number and pattern of network activity propagation was observed in Fmr1-KOs compared to wild-type recordings. This would again be expected to produce correlations that were less sensitive to averaging across trials compared to in wild-types.

Taken together therefore, data from this analysis suggest that correlated spiking activity recorded during cortical network activity is spatially restricted in Fmr1-KOs, such that network activity both engages a smaller area of cortical tissue, and is less capable of sustaining correlated neural firing during recurrent circuit activity. An intriguing hypothesis following this conclusion is that the elevated feedback inhibition demonstrated in
Layer 4 in this chapter additionally exerts a strong centre-surround inhibitory effect that excessively decorrelates excitatory network activity (Poulet and Petersen, 2008; Gentet et al., 2010; Haider et al., 2010; Middleton et al., 2012; Tetzlaff et al., 2012; Bernacchia and Wang, 2013; Graupner and Reyes, 2013; Sippy and Yuste, 2013). This might be additionally contribute to the depressed spatiotemporal propagation of MUA activity, as has been previously explored for “synfire chain” propagation (Reyes, 2003; Vogels and Abbott, 2005; Kumar et al., 2008; Marre et al., 2009; Long et al., 2010). Altered surround suppression also raises strong predictions for the fidelity of sparse, columnar network processing, with imminently testable effects on somatosensory cortical coding in vivo (reviewed with relevance in Casanova et al. 2003; Sachdev et al. 2012).

5.4. Discussion

Together, data presented in this chapter demonstrate abnormal timing, rate and trial-to-trial stability of TC-evoked P10/11 Fmr1-KO Layer 4 network activity from the perspective of both single-cell and multi-electrode array recordings. These results strongly implicate unbalanced inhibitory/excitatory synaptic transmission in the emergent Fmr1-KO network phenotype and offer insight into how unbalancing the strength and timing of this ratio can lead to pathophysiological network function.
5.4.1. Developmental state of TC-evoked network responses in P10-11 Fmr1-KOs

To what extent do the findings from P10-11 Fmr1-KO recordings in this chapter represent a delay in the maturation of cortical processing performed on TC inputs?

When compared against known developmental milestones for circuit activity from previous in vivo studies, Fmr1-KO recordings from the current P10-11 dataset do indeed share some similarities with immature circuit responses. In particular, cortical responses to TC burst stimulation in Fmr1-KO slice recordings showed elevated LFP gamma power and spatiotemporally restricted synaptic activity, which is consistent with an immature cortical response (in particular, see Colonnese et al. (2010), figures 2 and 5, and developmental maturation of cortical VSD evoked response in Yang et al. (2012), figure 1). This interpretation is also consistent with the reduced rate of MUA and in Layer 4, and slower firing of Ex. neurons from cell-attached Fmr1-KO recordings in the present study.

As explored by MEA recordings in this chapter, propagation of TC-evoked network activity in Fmr1-KO slices was spatiotemporally weaker and less diverse compared to in wild-types, despite slower and jittery single-neuron firing patterns in Layer 4, and unstable and poorly-timed feedback inhibition. The correlation between MUA in Layer 4, and in simultaneously active channels was also significantly reduced. It is likely that this is contributed to by a reduction in firing activity in surrounding channels, although the heavy averaging used in this analysis could additionally mask a phase shift. It is important to note that in context of the seemingly
divergent findings of Gireesh and Plenz (2008) who, show that maturation of network activity in somatosensory cortex is associated with an increase in correlated firing at gamma frequencies and those of Golshani et al. (2009) and Rochefort et al. (2009), where a developmental desynchronization was observed. The latter two studies compared synchrony of fluorescent calcium transients, imaged with a frame rate <10Hz, whereas extracellular activity was sampled at 20kHz, yielding sub-millisecond precision. Different processes as compared over these two timescales support signal synchrony.

A similar distinction is made between the findings of the current study and a recent report of reduced developmental desynchronization in Fmr1-KO barrel cortex (Gonçalves et al., 2013). The authors reported increased synchrony of Ca^{2+} transients between neurons in Layer 2/3 during slow-wave sleep, anaesthesia and quiet wakefulness. Using whole-cell recordings they also show an elevated firing probability during UP states, which is consistent with the trend towards increased participation probability observed in the current study for cell-attached recordings in Layer 4. They also report an increased firing rate during UP states. This is likely to contribute to the reported increase in population synchrony: Interneuronal correlation during spike trains increases in proportion to firing rate (de la Rocha et al., 2007). Interestingly, Gonçalves et al. (2013) report a reduced modulation of network synchrony by isoflurane anaesthesia in Fmr1-KOs compared to wild-types, suggesting that defects in GABAergic inputs could underlie altered population synchrony. Furthermore, the majority of signal in the reference calcium imaging studies arises in
superficial Layer 2/3, rather than in Layer 4 as in the current study. At P10-11 in wild-type brains, Layer 4 is in a more advanced developmental state compared to superficial layers, and development of recurrent inhibition in Layer 2/3 is sensitive to sensory deprivation (Shao et al., 2013). It will be interesting to establish if this is a secondary effect of mis-informative sensory relay from Layer 4. The distorted spike times reported in this study could disrupt the plasticity mechanisms involved in development of Layer 4 to Layer 2/3 connections – known to be sensitive to spike timing (Feldman, 2000). Perhaps this could similarly underlie altered anatomical development of these connections in Fmr1-KOs (Bureau et al., 2008).

Layer 4-2/3 spike timing precision is also maintained by feed-forward inhibition\(^61\), the development and plasticity of which is tightly modulated by whisker experience (House et al., 2011). A deeply insightful experiment would be to examine spike timing precision and plasticity in a layer-specific Fmr1-KO mouse, or even in on with specific genetic ablation of FMRP in supragranular layer interneurons. This would be technically difficult, although perhaps specifically targeting CGE-derived interneurons, which predominantly innervate supragranular layers, would be a useful strategy to disrupt inhibitory tone, if not FFI in Layer 2/3.

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\(^61\) Inhibition is not essential for STDP at this synapse in slice preparations, but modulates the magnitude of the effect (Feldman, 2000, Fig. 5).
6. Conclusions and Predictions

6.1. Main conclusions

6.1.1. Circuit manifestations of elevated intrinsic excitability

Together, the data from this study demonstrate far-reaching consequences of altered intrinsic properties, and this forms the basis for the main conclusion of the work. In Chapter 3, the elevated input resistance of Fmr1-KO excitatory Layer 4 neurons was shown to distort their responses to a variety of intrinsically applied current stimuli, in particular to sinusoidal waveforms. In Chapter 4 this was extended to study dynamic voltage responses to controlled synaptic stimulation, where Fmr1-KO Layer 4 neurons were found to show a hyperexcitable response to thalamocortical stimulation. This was partially constrained by stronger FFI in a subset of neurons, however, no relationship between the strength of FFI received by a given neuron and its intrinsic excitability was observed. Correspondingly, Fmr1-KO neurons were found to be more sensitive to high frequency thalamocortical stimulation, manifesting as excessive voltage summation. Since the elevated input resistance of Fmr1-KO Layer 4 neurons also increased their membrane time constants, excessive voltage summation in response to low frequency stimulation was observed explaining the ectopic recruitment of spiking activity in the Layer 4 network by low frequency inputs.
Computational modelling identified elevated input resistance as the most disruptive change in *Fmr1*-KOs from the perspective of thalamocortical temporal resolution. However, this phenotype also interacted strongly with altered timing and short-term synaptic kinetics in the FFI circuit to distort the emergent temporal filter and explain vulnerability to high frequency thalamocortical stimulation. Analysing the timing of network-sustained firing evoked by thalamocortical stimulation, spikes fired by Layer 4 excitatory neurons in *Fmr1*-KOs typically arrived later and at a slower rate, which is consistent with the findings from Chapter 3 on spikes evoked by direct depolarisation with injected current. This effect is furthermore consistent with the elevated input resistance and longer time constants of these cells.

### 6.1.2. Circuit manifestations of altered inhibition/excitation balance

As discussed in the introduction, altered balances between inhibition and excitation have been implicated in several aspects of pathology in FXS and ASD. However, to date, how this directly influences information processing has not been explored in detail. This study promotes a holistic approach to studying inhibitory and excitatory circuitry by additionally considering the intrinsic properties of neurons. Although subtle changes to the relative mean amplitudes of spontaneous inhibitory/excitatory currents were found in whole-cell recordings from Layer 4 neurons in Chapter 3, larger effects were apparent when evoked currents were investigated. Relative to excitatory synaptic currents, both the timing and amplitude of inhibitory currents were affected in *Fmr1*-KO recordings.
In Chapter 4, the timing of inhibitory feed-forward currents was identified as a key component of abnormal FFI in Fmr1-KOs, whilst the strength of FFI was a crucial factor in determining the overall thalamocortical frequency resolution. Synaptic kinetics and short-term plasticity played a smaller role by comparison.

Network-sustained feedback inhibitory currents were also distorted in Fmr1-KO recordings, both slower and with reduced timing fidelity compared to excitatory currents. These factors likely contributed to the lower spike timing precision observed in cell-attached recordings. Interestingly, despite the increased intrinsic excitability of Fmr1-KO neurons, their spike rates were slightly depressed relative to those in wild-types during network-sustained firing, while multiunit firing was significantly depressed and confined to a tighter spatial radius. From the current data, the largest contribution to this effect most likely arises from stronger feedback inhibition, which also manifests as a putative stronger inhibitory surround response.

Overall the network sustained cortical firing reported in Chapter 4 is depressed rather than hyperexcitable, further suggesting that excitatory network connectivity is immature in Fmr1-KOs at this age, whereas despite receiving statistically fewer connections from FS interneurons, maturation of inhibitory circuitry is less affected by comparison.

This conclusion is supported by proxy by the lower rate of spontaneous excitatory currents observed in whole-cell recordings in Chapter 3, although the connectivity structure of the Layer 4 Ex. network needs to be
examined in greater detail in these animals. The fine interactions between excitatory and inhibitory firing activity warrant further investigation, particularly given the slower membrane time constants of both cell types. Network simulations will prove a powerful tool for this purpose.

The extent to which the stronger gamma-band oscillatory power observed in *Fmr1*-KOs emerges from disrupted inhibitory circuit activity is also the subject of ongoing investigation. As discussed above, GABAergic circuit activity has been shown to critically support the emergence of gamma frequency content in extracellular field potentials by entraining synchronised firing of target cell populations (Cardin et al., 2009; Akam et al., 2012). The stronger gamma power as reported here is consistent with these previous findings, whereas the weaker power at higher frequencies is most likely to arise through weaker precise spike correlations. This could therefore be a secondary effect of impaired enforcement of spike timing by poorly timed inhibition.

6.1.3. **Thalamocortical and intracortical processing**

Previous studies have suggested that cortical network hyperexcitability underlies symptoms of hyperarousal, sensory hypersensitivity and the increased seizure propensity observed in FXS patients (Gibson et al., 2008; Gonçalves et al., 2013). However at the developmental time-point studied in this work, thalamocortical rather than intracortical activity appears to be the more hyperexcitable circuit component. Overall, the current data present a picture of excessive thalamocortical sensitivity, but under-developed (or, over-damped) intracortical responsiveness.
6.2. Are the results developmentally transient?

Compared to previous functional descriptions of FXS circuit activity, the largest difference presented by this work is the stronger In/Ex balance. At P14 and P28, Gibson et al. (2008) showed weaker feedback inhibition in the barrel cortex of Fmr1-KOs, whereas Gonçalves et al. (2013) interpreted reduced anaesthesia sensitivity of cortical firing state in P14-16 Fmr1-KO mice to weaker inhibitory activity. These studies were performed on older mice (and the latter focused on Layer II/III). It is thus plausible that a distinct developmental epoch, characterised by a relative lag in excitatory versus inhibitory development, precedes the cortical In/Ex balance situation presented in the previous studies. Given the importance of inhibitory circuit integration in the maturation of both thalamocortical (Daw et al., 2007a; Chittajallu and Isaac, 2010) and intracortical (Colonnese et al., 2010; Minlebaev et al., 2011) processing, more focus should therefore be placed on the early postnatal development of cortical circuits in models of ASD such the Fmr1-KO.

Interestingly, although diminished, the elevated input resistance observed in the current study at P10-11 was also previously reported by Gibson and colleagues (2008) at older ages. Given the far-reaching implications of elevated input resistance explored by this thesis, it is likely that a significant effect on circuit activity remains, and should not be regarded as developmentally transient or as a peripheral phenotype to circuit dysfunction in older animals.
6.3. Predicted consequences for sensory processing in *Fmr1-KOs*

It is important to speculate how the changes to functional cortical activation by thalamocortical stimuli will affect information processing, and by extension, somatosensory perception in *Fmr1-KO* mice and FXS individuals. Caution is urged however in translating the current findings in brain slices to sensory processing in the intact brain, and further generalising these finding to other cortical areas.

A prominent prediction is that brief touch will evoke a greater cortical response in *Fmr1-KOs*. This will arise due to greater integration of thalamocortical input. It is possible that during initial contact with objects (i.e. while strong FFI is still present) that texture sensitivity will be similar to, or even enhanced compared to wild-types due to initially stronger FFI. Consequently, it is conceivable that brief contact could provide more sensory information in *Fmr1-KOs* compared to wild-type littermates.

However, during ongoing sensory stimulation typical of prolonged surface contact experienced during tactile exploration, it is likely that less information will be available, and response will be curtailed and unreliable: Here, thalamocortical responses are typically depressed by ongoing stimulation, and representation of sensory input relies on amplified reverberating firing activity in Layer 4. From the present data, this is likely to be compromised in *Fmr1-KOs* such that prolonged contact is both less efficacious at activating cortical circuitry as well as sustained to a lesser extent. These predictions emerge from exaggerated depression of
thalamocortical input, and weaker excitatory but stronger inhibitory recurrent circuitry, respectively. Cortical responses are also predicted to be less reliable during sustained cortical input, but more robust during brief, initial contact. Consequently, Fmr1-KOs are predicted to outperform wild-type animals on tasks that require rapid sensory judgement, or discrimination of fine textures from brief contact. Conversely, they are expected to perform sub-par compared to wild-types on tasks that involve longer, more sustained sensory exploration, such as distinguishing frequencies of sustained tactile vibrations.

The predictions outlined above are of great interest and will be addressed using combined in vivo physiology and behavioural testing. Given that sensory behavioural therapy is considered a powerful alternative, or adjunctive therapy to pharmacology for ASD (reviewed in Cascio, 2010), better understanding of how sensory processing is distorted in these individuals will help improve these therapies. Furthermore, clarifying the mechanisms by which behavioural changes emerge from motifs of circuit-level physiological distortion will provide insight into common circuit processes that could underlie effects on higher cognitive areas of the ASD brain.

A final cautionary note is necessary here however: cortical microcircuits are both dynamic and highly interconnected. Due to the highly integrative and multi-scale nature of cortical circuit processing, a nuanced, holistic strategy is necessary when attempting to deconstruct the mechanisms and vulnerabilities of cortical circuit processing. As illustrated by this thesis, seemingly small circuit distributed effects can form cascades of
pathophysiology and lead to widespread disruptions in circuit function. Future work should incorporate functional and anatomical plasticity at multiple spatiotemporal scales, and should be inspired by the dynamic nature of cortical activity.
7. References cited

Abbeduto L, Hagerman RJ (1997) LANGUAGE AND COMMUNICATION IN FRAGILE X
SYNDROME. Ment Retard … 322:313–322.


Abbott LF, Varela JA, Sen K, Nelson SB (1997) Synaptic depression and cortical gain
control. Science (80-) 275.


Adesnik H, Scanziani M (2010) Lateral competition for cortical space by layer-specific

Adusei DC, Pacey LKK, Chen D, Hampson DR (2010) Early developmental alterations in
GABAergic protein expression in fragile X knockout mice. Neuropharmacology

Agmon A, Connors BW (1991) Thalamocortical responses of mouse somatosensory (barrel)


Ahissar E, Sosnik R, Haidarliu S (2000) Transformation from temporal to rate coding in a

Akam T, Oren I, Mantoan L, Ferenczi E, Kullmann DM (2012) Oscillatory dynamics in the

Akam TE, Kullmann DM (2010) Oscillations and filtering networks support flexible

Akam TE, Kullmann DM (2012) Efficient “communication through coherence” requires
oscillations structured to minimize interference between signals. PLoS Comput Biol
8:e1002760.

synapses displaying different dynamic properties in rat neocortex. Cereb cortex
16:386–393.

Angelo K, Racz E a, Pimentel D, Hundahl C, Hannibal J, Fleischmann A, Pichler B,
Margrie TW (2012) A biophysical signature of network affiliation and sensory

Ascoli G a et al. (2008) Petilla terminology: nomenclature of features of GABAergic

Ashby MC, Isaac JTR (2011) Maturation of a recurrent excitatory neocortical circuit by
experience-dependent unsilencing of newly formed dendritic spines. Neuron 70:510–
521.

Assisi C, Stopfer M, Bazhenov M (2012) Excitatory local interneurons enhance tuning of


400


Ornitz EM, Forsythe a B, de la Peña a (1973) Effect of vestibular and auditory stimulation on the REMs of REM sleep in autistic children. Arch Gen Psychiatry 29:786–791.


Steriade M, Nauiecz A (1993) A Novel Slow ( < 1 Hz ) Oscillation Depolarizing and Hyperpolarizing of Neocortical NATURAL. 73.


Wijetunge LS (2009) Role of mGlur5 and FMRP in Mouse Primary Somatosensory Cortex.


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*Table 1. Messenger RNAs associated with the FMRP-mRNP in Mouse Brain*

*Probe sets are ranked by the sum of the fold change values (set-P versus KD-1P) + (set-P versus Input) determined by MG-U74 microarray analysis.*

*N/A denotes Fmrp-mRNP-associated RNAs determined by MG-U74 not represented on the Mu19K chips.*

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Appendix 1 - mRNAs associated with the FMRP-mRNP in mouse brain, reproduced from Brown et al. (2001)
Appendix 2 A genotype-cooperative fuzzy c-means cluster analysis strategy for unsupervised classification of Layer 4 interneurons

Figure A2-1 Principle component analysis (PCA) of human-classified FS interneurons in P10/11 wild-type (blue) and Fmr1-KO (red) recordings. Data is from recordings presented in Figures 3-17 – 3-19.

a). Example recordings (top) and variance contributed by top 10 parameters (AP shape and intrinsic properties). PCA was applied to each genotype individually

b). Fraction of total variance explained (bars) and cumulative variance (lines) explained for recordings in a).
a

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b

![Graph showing variance explained vs. principal component number for Fmr1+/Y](image)

![Graph showing variance explained vs. principal component number for Fmr1-/-Y](image)
Figure A2-2 Co-variance between descriptive electrophysiological parameters for interneurons in wild-type recordings. Data is from recordings presented in Figures 3-17 – 3-19.

Redlabelled parameters (off-diagonal combinations with $R^2$ values $<0.8$) were removed from clustering/classification criteria for subsequent analysis.
Key
Threshold = spike @ rheobase
2xThreshold = spikes @ 2x rheobase
First = first spike shape
SS = steady-state spike shape
RELAX = Adaptation ratio

Pearson R² value between variables for Fmr1+/− dataset
Figure A2-3 FS and non-FS interneurons are more similar in P10/11 Fmr1-KO recordings compared to wild-type recordings.

Cluster analysis of FS and non-FS interneurons from Layer 4 P10/11 recordings based upon electrophysiological parameters shown in Figure A2-1.

a). Left and centre: Hierarchical clustering of wild-type and Fmr1-KO interneurons. The dendrograms show partitioning of neurons into clusters based upon separated by Mahalanobis distance based upon electrophysiological characterisation. Colours indicate putative cluster membership based upon subjective human classification of FS and non-FS membership.

Right: Silhouette values (a metric of cluster separation) for dendrograms show on left as a function of normalised branch height.

b). Blind separation of neurons into 2 clusters using k-means clustering of wild-type (blue) and Fmr1-KO (red) neurons, with genotypes treated independently. Data are projected into top 3 principle components. Circles and triangles indicate putative FS and non-FS interneurons, respectively. Nets indicate closest fitting spheroid convex hull, highlighting boundaries of cluster membership. Note weaker rejection of putatively non-FS cells in the Fmr1-KO case. Right: Partition coefficient for each genotype for number of clusters k=1–10.
Figure A2-4 Unsupervised and supervised strategies for Fuzzy c-means classification of wild-type and Fmr1-KO interneurons into FS or non-FS subtypes using electrophysiological parameters shown in Figure A2-2 and data from Figures 3-17 – 3-19.

a) 2-dimensional Sammon projection showing soft classification of wild-type interneurons into c=2 clusters. Black isobars indicate odds ratios for membership into each category. Dotted line indicates membership nullcline (odds-ratio = 50:50). Green points are unclassified neurons (membership odds-ratio cutoff >0.2). Asterisks are cluster centroids.

b) As in a) but for Fmr1-KO interneurons categorised independently. Note poor discriminability between categories and impaired performance (16 unclassified recordings).

c) Supervised classification of Fmr1-KO interneurons into wild-type (training set) clusters established in a). Note improved discriminability (13 unclassified recordings). N.B. Sammon map re-orientates for best display of new data.
Figure A2-5 Comparison of performance of strategy shown in Figure A2-4 as a function of membership stringency criterion. Ground truth was taken as human classification scheme - for all interneurons, even ambiguous to expert observer.
Figure a: Fraction in agreement with human classification.

Figure b: Human vs. machine: disagreement bias by cell type.

Figure c: Fraction unclassified.

Figure d: Unclassification: bias towards FS.

Inclusion threshold (odds-ratio between cluster membership)
Figure A2-5 Validation of classification performance: strategy shown in Figure A2-4a and c against human expert classifier using odds-ratio membership cut-off of >0.2 for each of wild-type (blue, a) and Fmr1-KO evaluated in wild-type clusters (red, b). no APs fired/500ms was chosen as a performance example since this dimension explains the most variance in wild-type recordings, and is the 3rd best descriptor for independently evaluated Fmr1-KO neurons (Figure A2-1).