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MOLECULAR MECHANISMS OF NEURONAL HOMEOSTASIS IN VIVO

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Doctor of Philosophy
School of biological sciences
University of Edinburgh
2016
Sang soo Seo:
Molecular mechanisms of neuronal homeostasis in vivo
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LAY SUMMARY

Neuronal homeostasis is a process whereby a neurone regulates itself to maintain a constant level of activity. This is important as too much or too little activity leads to death of neurons. Moreover, there is growing evidence that homeostatic regulation is impaired in neurological diseases such as autism, Rett syndrome and schizophrenia. However, most of our knowledge about neuronal homeostasis comes from experiments using cell cultures and we know little about neuronal homeostasis in the intact brain. Recent theories of neuronal homeostasis suggest important roles for regulation of gene expression, but this too has received little experimental attention. Identification of genes whose expression changes could be important for developing drug targets for neurological diseases.

In this thesis I have established an experimental system for investigating homeostatic adaptation in the intact mouse brain. I used stereotaxic surgery to deliver viruses into a brain region called the hippocampus. The virus was designed to genetically manipulate neurones and suppress their activity. I then prepared thin slices of the mouse brain and recorded the electrical properties of the neurons through a method called patch-clamp. Through these experiments I showed that the genetic manipulation was successful as the neurones showed predicted changes in their excitability. Importantly, two weeks after surgery, I also identified changes to increase neuronal activity, indicating that neurones that I genetically manipulated had homeostatically adapted. I showed that the mechanism used for the adaptation differs in different types of neurons.

I then used a method called fluorescent activated cell sorting to isolate the genetically manipulated cells from unaffected surrounding cells. I isolated the mRNA from the manipulated cells and compared the expressed genes to those of non-manipulated cells. To this end I used a technique called RNAseq, which sequences the mRNAs in the sample in a quantitative way. The results identify a number of genes with expression that has changed during homeostatic process. Several of these genes have not previously been associated with homeostatic process. In total, this work provides insight into the mechanisms of homeostasis in the intact brain and identifies novel gene candidates that may be involved in homeostatic processes.
ABSTRACT

Homeostatic plasticity is important in neurobiology for stabilising neuronal networks in the face of Hebbian forms of synaptic plasticity that are thought to mediate memory storage. Impairment of homeostatic plasticity has also been implicated in neurological diseases such as Rett syndrome and fragile X syndrome. Homeostatic plasticity can be achieved through scaling of the strength of synaptic connections between neurones or by changes in intrinsic excitability. While homeostatic plasticity has been studied mainly using in vitro preparations, it is for the most part not known whether changes of neural activity in vivo induce homeostatic changes. The molecular pathway responsible for homeostatic plasticity still remains unclear.

In this thesis, I have used stereotaxic surgery to over express Kir2.1, an inwardly rectifying potassium channel, in vivo in the brains of adult mice. I show that the expression of Kir2.1 through adeno-associated virus (AAV) does not cause any adverse effects in the dentate gyrus nor the CA1 of the mouse hippocampus. I go on to use slice patch clamp methods to measure the change in electrical properties of granule cells in the dentate gyrus and pyramidal cells in CA1 caused by expression of Kir2.1. I show that the excitability of neurones expressing Kir2.1 was reduced compared to control neurones. By 2 weeks after virus injection the neurones showed homeostatic plasticity in response to Kir2.1 over expression. Interestingly, the mechanism of adaptation was different in different types of cells; dentate gyrus granule cells adapted through change in their intrinsic excitability, whereas CA1 pyramidal cells adapted by modifying the strength of their synaptic inputs.

To establish whether induction of homeostatic plasticity is associated with changes in gene expression I used fluorescent activated cell sorting (FACs) to isolate pure population of neurones infected with viruses. I then sequenced RNA extracted from neurones expressing Kir2.1 and control neurones. Analysis of the RNAseq data revealed molecular candidates involved in homeostatic plasticity.

In summary, I show that Kir2.1 over expression causes change in excitability and subsequent homeostatic plasticity in vivo. The mechanism of adaptation differs between cell types. RNAseq results identify novel candidates for future investigation.
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I would like to thank the members of QMRI flow facility for their assistance with FACs experiments, Dr. Trudi Gillespie for help with the microscope works, Vivian Allison and Louise Dunn for assistance during histology. Also Prof. Jean-Marc Fritschy for his collaboration.

My family and friends for all their support. My dad for guidance and wisdom, my mum for her love and my sister for her friendship.

For from him and through him and for him are all things. To him be the glory forever.
DECLARATION

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

_Edinburgh, 2016_

Sang soo Seo, May 9, 2016
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ABBREVIATIONS

aCSF  artificial cerebrospinal fluid
PBS  phosphate buffered saline
DEPC  diethylpyrocarbonate
DG  dentate gyrus
PFA  paraformaldehyde
TX100  triton X 100
NGS  normal goat serum
FJC  fluoro Jade C
BSA  bovine serum albumin
FCS  fetal calf serum
FACs  fluorescence activated cell sorting
H and E  hematoxylin and eosin
AAV  adeno-associated virus
FPKM  fragments Per Kilobase of Exon per Million reads
LTP  long term potentiation
LTD  long term depression
TTX  tetrodotoxin
BCM theory  Bienenstock, Cooper and Munro theory
INTRODUCTION

Synapses are specialised structures where neurones communicate with other neurones. Synapse consists of pre and post synaptic terminals. Neurotransmitters are released from the presynaptic terminal and are recognised by their receptors in the postsynaptic terminal. This then produces specific changes in the postsynaptic cell giving rise to a response to the presynaptic signal. A neurone would receive synaptic connections from a large number of other neurones and would also send synaptic outputs to a large number of other neurones. These neuronal connection forms the bases of neuronal networks. The activity of the neuronal network depends on collective synaptic activity levels of each neurone in the network. The activity of each neurone is determined by the inputs from other neurones and the intrinsic excitability of the neurone.

The neuronal network is often stated to be plastic, i.e. able to change. This is a very important characteristic of neuronal networks which is responsible for formation of memory and computation. Plasticity causes changes in the network properties which provides the capacity information storage.

This plasticity of neuronal network, especially through the synapses, has been well documented (Huganir and Nicoll (2013), Turrigiano (2008)). Well studied durable form of plasticity is hebbian plasticity. This involves long lasting potentiation or depression of synapses. This is an important form of plasticity which forms the basis of memory. Another important form of plasticity is homeostatic plasticity. Homeostatic plasticity involves change in the synaptic strength or the intrinsic excitability in such a way to maintain a stable firing rate in a neurone. These two distinctive plastic mechanisms interact to allow computational capacity in the neuronal network as well as maintaining stability of the network activity.

Homeostatic plasticity is important for neuronal health and for maintaining stable neuronal network (Ramocki and Zoghbi (2008)). Therefore it has been subjected to intense investigation. However, there are still no coherent understandings of physiological and molecular events leading to homeostatic plasticity in vivo. In this thesis I will aim to determine the physiological mechanisms and the responsible molecular
changes in the adult mouse hippocampus \textit{in vivo} during plasticity.

In this chapter, I will discuss some of the key works which have contributed to our understanding of the mechanisms of hebbian form of plasticity and homeostatic plasticity. I will then state the aim of the thesis and give a brief reasoning behind the experimental approach. There will also be a detailed introduction behind each experiment in the chapters where the results are presented.

\section*{1.1 Hebbian Synaptic Plasticity}

Neurones in the brain are organised into neuronal networks through their connections via synapses. As the synaptic connections are the core of neuronal signalling, these synaptic connections needs to be plastic for cognitive capacity and information storage. In 1949, D.O. Hebb proposed a theoretical model in his book ‘The Organisation of Behaviour’ which predicted the basic theory behind LTP (long term potentiation) and LTD (long term depression). LTP describes a process which there is a lasting potentiated in the synaptic strength and LTD is a process where the synapses are depressed in the same manner. The two mechanisms are often collectively named Hebbian plasticity. Both has been suggested as a cellular basis for memory due to its ability to have long lasting changes in the synapses (Huganir and Nicoll (2013)).

The first experimental evidence of LTP came from T. V. P. Bliss and T. LoMo (1973) where the authors observed an increase in ‘population’ EPSP (excitatory post synaptic potential), later termed field EPSP, in rabbit DG cells after stimulation of perforant pathway, an input pathway to the hippocampus from entorhinal cortex. Later work has shown that this LTP requires NMDA receptor activation (Collingridge et al. (1983)) which then results in an influx of calcium (Lynch et al. (1983)) to cause LTP. Morris et al. (1986) also showed that blocking NMDA receptor with infusion of AP5, a selective antagonist of NMDA receptor, causes a loss in LTP and also disrupts the formation of spacial memory. This suggests an important role of LTP in memory formation. Initial works on LTP are reviewed in Nicoll et al. (1988). After the initial experiments, a large amounts of literature has been produced looking more in depth the mechanisms of LTP (reviewed in Huganir and Nicoll (2013), Malinow et al. (2000)).

LTD was first demonstrated in hippocampal CA1 pyramidal cells by Dudek and Bear (1992). The authors used a low frequency stimulation protocol to induce lasting depression in the synapses. Since the initial demonstration in CA1 pyramidal
cells, wide range of literature was also produced investigating the mechanism of LTD and implications for memory formation (reviewed in Huganir and Nicoll (2013), Collingridge et al. (2010)).

LTP and LTD research has shown evidences for a maintained synaptic alterations in the neuronal network level in response to neuronal activity or experience. This maintained alterations in the synapses has a massive capacity for information storing as there are billions of synapses in the brain. There are also direct evidences for its involvement in memory formation and links with intellectual disabilities such as autism (Huber et al. (2002), Bear et al. (2004), Auerbach et al. (2011)).

1.1.1 BCM theory

Experimental evidences for LTP and LTD support Hebb’s theory describing potentiation of high frequency stimulated synapses and depression of the low frequency stimulated synapses. However as this theory is based on a positive feed back loop it would allow run away excitation in the potentiated synapses and complete silencing of the depressed synapses.

To overcome this theoretical shortcoming, Bienenstock et al. (1982) proposed a revised theory, BCM theory (Bienenstock, Cooper, and Munro theory), concerning cortical synapses. The theory states that a synapse will potentiate as it is stimulated above a certain activity threshold and will depress as it is stimulated less than the threshold. This part is exactly the same as the Hebbian theory but in BCM theory the threshold value is also subject to change depending on the level activity and as the synapse is potentiated the threshold value would increase making the synapse more likely to depress and as the synapse is depressed the threshold value would decrease making the synapse more likely to potentiate. This theory has also been denoted as “metaplasticity” as the aspect of plasticity (threshold at which plasticity occurs) is also subject to other plastic mechanisms.

Dudek and Bear (1992) directly tested this theory in rat visual cortex. Dudek and Bear (1992) prepared acute slices from light deprived rats and in control rats and induced LTP and LTD in stimulating with various frequencies. The results shows clear drop in the LTP threshold in light deprived rats thus providing a strong evidence for the theory. Subsequent work on this theory was recently reviewed in Cooper and
Bear (2012). Dysfunction of this mechanism has been linked with behaviour and neurological diseases (Hulme et al. (2013)).

1.2 HOMEOSTATIC PLASTICITY

Initial theoretical work of neuronal homeostatic plasticity

Another theoretical solution for the issue of instability caused by hebbian plasticity alone was suggested by LeMasson et al. (1993) who predicted a negative feedback homeostatic loop in regulation of neuronal activity based on a neuronal model (Marder and Goaillard (2006)). This neuronal model consists of fast Na⁺, delayed rectifier K⁺ current, fast transient K⁺ current, slow transient K⁺ current, hyperpolarisation activated inward current, transient Ca²⁺ current, Ca²⁺ dependent K⁺ current and a passive leak current. The 7 currents (excluding the passive leak current) makes up the conductance of the neurone. The model shows that the intracellular concentration of Ca²⁺ correlates with the neuronal activity given the biological channel properties of the 7 channels. Also the model demonstrated a change in intrinsic conductance in responsible to activity suggesting a change in intrinsic conductance in response to activity and suggested Ca²⁺ as a sensor for neuronal activity. This theoretical idea is distinguished from the Hebb’s theory and BCM theory in that it predicts change in the conductance in a whole neurone rather than a single synapse in response to neuronal activity. Hebb’s theory and BCM theory has implications in integration of neuronal networks. As the network undergoes hebbian plasticity (explained by both Hebb’s theory and BCM theory) the precise weight of each synapses changes changing the ‘wiring’ of the neuronal circuit. This change of wiring would result in the change in the total level of neuronal activity. The homeostatic plasticity, predicted by LeMasson et al. (1993), would change the excitability of the whole neurone so that the total level of activity is constant but also conserving the specific wiring of the neuronal circuit. Stable level of activity in individual neurones will ultimately result in stable network activity. The combination of two mechanism allows both computational capacity as well as stability of the network.

Demonstration of homeostatic plasticity in biological systems

The theory led to experimental work examining changes in the neuronal conductance after manipulation of neuronal activity (Turrigiano et al. (1998)). The authors used pharmacological manipulations to change neuronal activity in cortical cultures and measured all postsynaptic synaptic activity in a post synaptic neurone with patch
clamp method in neuronal cultures.

The authors found that when neuronal activity is blocked with TTX infusion, the amplitude of the miniature excitatory postsynaptic currents (mEPSC) was increased, indicating scaling up of the synaptic strength. Blocking GABA mediated inhibitory drive with bicuculline resulted in scaling down of mEPSC amplitude. The authors also confirmed the homeostatic effects of the synaptic scaling by demonstrating that the firing rate after bicuculline application for 48 hours were similar to firing rates measured in control conditions. Acute application of bicuculline significantly increased the firing rate of the neurones. This global change in synaptic strength of a neurone is important for computation as it would maintain the synaptic specificity in strengths. During LTP and LTD, synapses are potentiated or depressed according to experience thus storing information. However as potentiation of synapse increases the likelihood of the postsynaptic neurone to be activated it would increase the the chance of other synapses to be activated also. This would lead to further LTP in other synapses which then increases the likelihood even more and vice versa. This is prevented by the global weakening of all synapses. As the effect is global this would keep the specificity of between the synapses which has undergone LTP from other synapses thus maintaining stored information as well as maintaining a stable network (Turrigiano (2008)).

Since the discovery of homeostatic plasticity in 1998 (Turrigiano et al. (1998)), much research has been carried out to determine the cellular mechanism of homeostatic plasticity. There has been two main mechanisms of homeostatic plasticity that has been suggested. I will first present evidences for a mechanism named synaptic scaling, then I will present evidences for a mechanism named change in intrinsic properties.

1.2.1 Synaptic scaling mechanism of homeostatic plasticity

Initial works detected a bidirectional scaling of mEPSC amplitude in response to chronic change of neuronal activity (Turrigiano et al. (1998), O’Brien et al. (1998), Lissin et al. (1998)). As mEPSC is a measure of synaptic activity in a cell, increase in the amplitude of mEPSC would mean scaling up of the synaptic gain in response to each synaptic activity thus increasing the overall post synaptic activity. This phenomena was named synaptic scaling and it is the most well studied form of homeostatic plasticity in central neurones (Turrigiano (2008), Turrigiano (2012)). Two recent stud-
ies (Hengen et al. (2013) and Keck et al. (2013)) demonstrates homeostatic adaptation in visual cortex in wake behaving mice through synaptic scaling.

1.2.1.1 Activity dependent regulation of postsynaptic strength

AMPA receptors are tetramers made up of 4 possible subunits (GluR1 to GluR4). It usually exists in hetromers containing GluR2 in excitatory neurones. GluR2 lacking AMPA receptors can be found especially in GABAergic neurones and, unlike GluR2 containing channels, they are Ca²⁺ permeable channels (Isaac et al. (2007)).

Turrigiano et al. (1998) demonstrated that blocking of neuronal activity with TTX or enhancing of neuronal activity with bicuculline caused bidirectional homeostatic plasticity resulting in reseting of neuronal activity level to a set point. The authors also showed that this was achieved by changing the amplitude of mEPSC. This work was confirmed by studies showing activity dependent change in the expression level of GluR1, a sub unit of AMPA receptor, suggesting that this change in the amplitude of mEPSC is due to the change in AMPA currents (O’Brien et al. (1998), Lissin et al. (1998), Shepherd et al. (2006), Ju et al. (2004)). Manipulation of activity did not result in detectable change in the number of synapses further confirming the change is in the AMPA receptors and its strength in the postsynaptic neurone (O’Brien et al. (1998)).

Role of AMPA receptor subtypes during synaptic scaling

The subtype specific scaling of AMPA receptors has been debated for a long time (Turrigiano (2012), Lee (2012)). Activity dependent scaling of GluR1 has been largely agreed on but activity dependent change in GluR2 has been debated. Shepherd et al. (2006), Thiagarajan et al. (2005) and Sutton et al. (2006) reports no change in GluR2 in response to activity blockade but O’Brien et al. (1998), Wierenga et al. (2005), Ibata et al. (2008) Diering et al. (2014) reports changes in GluR2 and GluR3 in response to activity blockade. Moreover, knock down of endogenous GluR2 resulted in impairment of synaptic scaling (Gainey et al. (2009)) indicating the importance of GluR2 in synaptic scaling. The precise reason for this difference is still unclear but this may be due to the difference in experimental preparation as there are studies showing different mechanisms of homeostatic regulation dependent on the developmental stage (Echegoyen et al. (2007), Desai et al. (2002), Burrone et al. (2002)) and cell types (Kim and Tsien (2008)).
Local and global synaptic scaling

One of the important questions regarding homeostatic plasticity is how the neurone would sense the change in activity in a neural network. It would be possible that for a given neurone, embedded in the neuronal circuit, the activity change would only be confined to a small area in the dendrite where it receives inputs from specific neurones. As the initial work has relied on a complete alteration of activity, which may not be physiologically common, it is important to know what activity change in small group of synapses would do to the neuronal firing. To address this question, Ibata et al. (2008) used local application of TTX or NBQX/AP5 to alter neuronal activity and measured AMPA receptor accumulation. The authors found that local application of TTX or NBQX/AP5 to the dendritic region did not alter the level of AMPA receptors. Interestingly local application of TTX to soma up regulated dendritic GluR2 levels indicating that the neurones are sensing global activity through the soma rather than local dendritic activity for inducing homeostatic plasticity. This agrees with the theory proposed in Turrigiano (2008) that local change in synapses will induce hebbian form of plasticity and as the global activity starts to change the neurone will scale the synapses globally to maintain synaptic specificity.

As well as slow plasticity in response to blockade of global activity of the cell, there also seems to be a fast homeostatic plasticity in response to local alteration of receptor activity. The TTX induced scaling up of mEPSC amplitude are observed at least 18 hours after TTX application (Sutton et al. (2006), Turrigiano et al. (1998)). However scaling up of mEPSC amplitude can be observed when NMDA receptor is blocked by application of AP5, selective antagonist of NMDA receptor, for 1 hour during TTX application. Interestingly, local application of AP5 at the dendrites induced local scaling up of GluR1 through eukaryotic elongation factor-2 (eEF2) dependent local protein translation (Sutton et al. (2006), Sutton et al. (2007)). As stated above, GluR2 lacking AMPA receptors are Ca2+ permeable. Up regulation of local GluR1 but not GluR2 in NMDA receptor blocking would serve to maintain Ca2+ homeostasis in local dendrites (Sutton et al. (2006)). Interestingly without the global blockade AP5 did not have any scaling effect on GluR1 nor GluR2. However NBQX, a selective AMPA receptor antagonist, scaled up GluR1 (Thiagarajan et al. (2005)). This also indicates that there needs to be suppression of action potentials for NMDA blockade to lead to synaptic scaling. Ju et al. (2004) also demonstrated that newly synthesised GluR1 can be fluorescently labelled in the isolated dendrites. This indicates that the GluR1 subunit which is up regulated upon the blockade of activity can also by synthesised
locally.

Interestingly, there is also evidence for homeostatic adaptation at single synapse level. Hou and Zhang (2008) used inwardly rectifying potassium channel Kir2.1 with YFP tagged synapsin and transfected the neuronal culture sparsely. Overexpression of Kir2.1 causes changes in membrane conductance and resting membrane potential thus decreasing firing in the transfected neurone. YFP tagged synapsin allows visualisation of synapses that the Kir2.1 expressing neurone makes to other cells in the circuit. By studying post synapses from a neurone with activity deprived pre synapse or normal pre synapse, the authors showed that there are increase in the GluR1 levels in the post synapses with activity deprived pre synapses showing homeostatic regulation occurs at single synapse level. The neurone that over expressed Kir2.1 did not show any global increase in both GluR1 and GluR2. How this single synapse homeostatic mechanism relates to the global homeostatic mechanism remains unclear.

**AMPA receptor trafficking during synaptic scaling**

Trafficking of AMPA receptors can be one of the easy and quick ways to scale excitatory strength of the cell. This trafficking mechanism is also involved in Hebbian form of synaptic plasticity (Malinow and Malenka (2002)). Ju et al. (2004) has also shown that locally synthesised AMPA receptors are trafficked to the synapses. There are also evidences that TNFα increases the surface AMPA receptors but not internalised AMPA receptors (Stellwagen et al. (2005)) and there is endocytosis of AMPA receptors in scaling down in response to increased activity (Hou et al. (2011)). The trafficking of AMPA receptors in homeostatic settings are thought to be mediated by Arc signalling (Shepherd et al. (2006)).

**Conclusion**

Studies presented in this section has investigated the physiological mechanism which induces various form of postsynaptic homeostatic plasticity. The precise mechanism of plasticity can vary from synapse specific homeostatic synaptic scaling to global plasticity. It can also be caused by global blockade or local dendritic block of NMDA receptors or single synapse suppression. The type of plasticity also depends on the cell type and developmental stage of the neurone. The meaning of this variability in the mechanism of plasticity in homeostatic plasticity in vivo remains unclear.
1.2.1.2 Activity dependent regulation of presynaptic strength

As well as changes in postsynaptic strength the network may homeostatically adapt to perturbation of activity by altering presynaptic strength. This presynaptic increase can be measured in the post synaptic neurone through measuring frequency of mEPSC events. As mEPSC is a read out of individual synaptic events, the frequency would be a read out of prevalence of synaptic events. This may be due to increased release probability of neurotransmitters in the presynapse, increase number of the synapse or even from un-silencing of silence synapses (Kerchner and Nicoll (2008)) which would involve post synaptic changes. However, the unsilencing is yet to be confirmed in homeostatic plasticity setting.

Developmental stage specific mechanism of synaptic changes

Burrone et al. (2002) have used over expression of Kir2.1, a subtype of inwardly rectifying potassium channel, to suppress activity of pyramidal cells and to induce homeostatic adaptation. This suppression of activity resulted in low firing frequency of Kir2.1 transfected neurone initially but the firing frequency of the neurone returned to the base line level after 4 days indicating homeostatic adaptation. Interestingly, in mature neurones, the amplitude of mEPSC from the transfected neurones were unchanged but the frequency was increased. This is opposing the observation from many studies which showed unchanged frequency with increased amplitude (Turrigiano et al. (1998), Gainey et al. (2015)). In Burrone et al. (2002) the number of synapses did not change in response to Kir2.1 transfection but the size of presynapses were significantly increased indicating that the higher frequency of mEPSC is due to change in probability of neurotransmitter release and not the increase in the number of synapses (but see Murthy et al. (2001)). Interestingly, TTX application, which has been used in studies where no changes in mEPSC frequency has been observed (Turrigiano et al. (1998), Gainey et al. (2015)), has shown mEPSC amplitude and frequency changes in older cultures but not in young cultures (Wierenga et al. (2006)) indicating that the change in presynapses are only observed in adult neurones. In line with this, Burrone et al. (2002) also observed homeostatic adaptation in 10 day old cultures only, synaptically mature cultures, and no homeostatic adaptation in 2-5 day old cultures even after 10 days of transfection of Kir2.1. Moreover, this increase in the frequency but not amplitude was also observed in vivo adult CA1 pyramidal cells following block of pre- and postsynaptic activity with TTX. However at juvenile CA1 both the amplitude and the frequency was scaled up in response to TTX (Echegoyen et al. (2007)).
Branco et al. (2008) demonstrated that the release probability of the synapses are regulated in an activity dependent manner. Also this regulation was spatially selective as local stimulation of dendrites only altered the release probability in near-by synapses. Others have demonstrated the same phenomena as well as probing into the molecular mechanisms of this release probability change (Kim and Ryan (2010), Weyhersmüller et al. (2011)). This change in release probability is also very well documented in neuromuscular junctions (for review see Davis and Müller (2015)).

The pre and post synaptic mechanisms can be correlated with activity (Tokuoka and Goda (2008)). This indicates that two mechanisms may be working together to maintain stable network activity in some preparations.

1.2.1.3 Inhibitory synapses

Excitatory synapses are regulated in an activity dependent manner to maintain homeostasis in neuronal network. Another strategy that a neuronal circuit might used to maintain stable network activity is by altering the inhibitory drive in the neuronal network. Modelling work has also shown that plasticity of inhibition is important for maintenance of neural circuit activity (Vogels et al. (2011)).

Early work from Rutherford et al. (1998) showed that there were no scaling of synaptic strength in interneurones in response to TTX. However, BDNF, a molecule suggested to be involved in scaling synaptic strength, was able to scale both excitatory synapses from pyramidal cells and inhibitory synapses from bipolar interneurons (Rutherford et al. (1998)). This result was quickly followed by wide range of studies showing activity dependent regulation of scaling of inhibitory synapses accompanied with change in immunoreactivity of GAD65 (Kilman et al. (2002), Hartman et al. (2006), Swanwick et al. (2006)). Importantly, the homeostatic scaling effect seems to be interneuron subtype specific (Bartley et al. (2008)), even scaling up of some type of inhibitory synapses in response to activity blockade has been observed which seem to oppose homeostatic regulation (Kim and Alger (2010)). Plasticity of inhibition changes dependent on developmental stage (Echegoyen et al. (2007)). In addition to GAD65, GAD67 is also involved in scaling of inhibitory synapses (Lau and Murthy (2012)).

1.2.2 Change in intrinsic properties

Another homeostatic adaptation which has been widely studied is change in intrinsic properties (Zhang and Linden (2003), Marder and Goaillard (2006), Turrigiano
(2011)). Different to synaptic scaling, which scales the input of activity to a neurone by scaling up or down of pre and post synapses, change in intrinsic electrical properties leads to changes in how much the postsynaptic cell reacts to a synaptic signal from presynaptic cell.

This idea has received much attention in neuronal network models. The model which lead to Turrigiano et al. (1998) predicted changes in the ion channel composition (intrinsic properties) in response to activity change (LeMasson et al. (1993)). Also a model predicted that by changing ion channel compositions of neurones a stable neuronal network can be generated consisting of neurones with firing which resembles patterns of various neuronal types (O’Leary et al. (2014)). Change in intrinsic properties are also suggested to be important for computational capacity of a neuronal network (Naudé et al. (2013)).

It has been quickly realised that the experimental paradigm used to induce synaptic scaling also induces changes in conductance for various ions thus changing intrinsic properties of the cell (Desai et al. (1999)). This homeostasis through changes in intrinsic properties is also observed in vivo in monocular deprived animals (Nataraj et al. (2010)). As well as homeostasis of neuronal firing it is also important for synchrony in neuronal networks (Cudmore et al. (2010)). Importantly, in agreement with the network models, this change in intrinsic properties seem to involve some sensing of Ca$^{2+}$ as CAMK2 is involved in the induction of change in intrinsic properties (Nelson et al. (2005), Marder and Goaillard (2006)). There has been examples where homeostatic plasticity in synapses and intrinsic properties are observed together suggesting that both mechanism can act together to compensate for the change (Howard et al. (2014), Tadros et al. (2014)).

Another interesting regulation of intrinsic properties comes not from the change in the amount of expression of ion channels but from the spatial alteration of ion channel expression. Axon initial segment (AIS) is a specialised structure at the start of an axon which is important for spike initiation (Yoshimura and Rasband (2014)). Upon change in activation AIS has been shown to elongate and translocate along the axon to homeostatically change excitability of the neurone (Kuba et al. (2010), Grubb and Burrone (2010)).

Work presented above demonstrates complex nature of homeostatic plasticity. There seems to be a number of strategies for adaptation where one or more mechanisms may be employed simultaneously by a neurone. The choice a neurone makes may
depend on the developmental stage of the neurone or the cell type of the neurone. Also, as neurones exists in a network adaptation may be a primary effect from the experimental manipulation or from the secondary effect from the change in neuronal activity of other cells in the network.

### 1.2.3 Molecular mechanisms of homeostatic plasticity

The physiological studies summarised above made much progress in explaining some of the fundamental mechanisms of homeostatic plasticity including AMPA receptor regulation, inhibitory synapse regulation, change in vesicle release probability and changes in intrinsic ion channel expression levels. Many studies have also been performed to investigate the molecular mechanisms of this process. When the level of neuronal activity is perturbed a neurone would sense the change through a molecule or various molecules. After the initial sensing of the change a neurone must initiate some molecular process to change the expression of synapses or ions channels. A coherent molecular mechanism from the initial sensing of the change, initiation and the maintenance of the change is not yet established (Siddoway et al. (2014), Turrigiano (2011), Turrigiano (2012)). However, there are many candidates which have been shown to play a role in homeostatic plasticity (Siddoway et al. (2014), Turrigiano (2011), Turrigiano (2012)). Here I will review the main molecular pathways revealed up to date.

#### 1.2.3.1 BDNF

Rutherford et al. (1998) showed that BDNF blocks the increase in mEPSC amplitude seen after TTX infusion in a dose response manner. As BDNF is also expressed in an activity dependent manner (Lu (2003)), BDNF is suggested as a molecule involved in synaptic scaling. However BDNF incubation alone did not scale synapses in pyramidal cells. Moreover blocking BDNF signalling by blocking TrkB receptors did not alter mEPSC scaling in response to elevated KCl in the culturing media (Leslie et al. (2001)). In inhibitory synapses, application of BDNF increases the amplitude of miniature inhibitory post synaptic current (mIPSC) and increased synaptic size of GAD65 indicating that BDNF may have a role in scaling inhibitory synapses in response to activity (Rutherford et al. (1998), Swanwick et al. (2006)) indicating that BDNF can function in different cell types to homeostatically regulate neuronal activity.

BDNF also stimulates the ERK1/2 MAP Kinase cascade thus activating mitogen and stress activated protein kinase 1(MSK1). Mice with inactivated MSK1 shows impaired synaptic adaptation in vivo and in vitro. This has also been linked with
dysregulation expression of activity-regulated cytoskeleton-associated protein/activity regulated gene 3.1 (Arc) which is also linked with synaptic scaling (Corrêa et al. (2012)).

1.2.3.2 Tumor necrosis factor α (TNFα)

Glial cells are the supporting cells in the nervous system. Recently there has been evidences of glia affecting synaptic function of neurones (Eroglu and Barres (2010)). TNFα is a molecule released from astrocytes (Harrison et al. (2007)). Beattie et al. (2002) demonstrated that incubation with TNFα in neuronal culture can increase the frequency of mEPSC. Blocking TNFα also decreased the frequency and amplitude of mEPSCs. The decrease in frequency was also observed in acute slices. The increase in the amplitude of mEPSC in response to TNFα was also observed (Stellwagen et al. (2005), Stellwagen and Malenka (2006)). This increase was GluR1 specific with unchanged GluR2 levels (Stellwagen et al. (2005)). TNFα also decreases the surface expression of GABARs and increase the internalisation of GABARs (Stellwagen et al. (2005)) thus decreasing the mIPSC (Stellwagen and Malenka (2006)). This process is dependent on a downstream signalling pathway de phosphorylating GABAA receptor (Pribiag and Stellwagen (2013)). The source of TNFα seems to be solely from glial cells as culture systems which has neurones from TNFα KO mice with WT glia showed no impairment of synaptic scaling where as systems with WT neurones with TNFα KO glia showed impaired synaptic scaling (Stellwagen and Malenka (2006)).

Stellwagen and Malenka (2006) found that 24hr incubation with TNFα dud not change the surface expression of AMPAR but the effect was only visible after 48hrs. The authors concluded that this is due to a slow build up of TNFα in response to change in activity. However synaptic scaling has been observed at earlier time points even after 4 hours after activity blockade (Turrigiano et al. (1998), Ibata et al. (2008)). Thus it has been suggested that TNFα is responsible for the maintenance of synaptic scaling but not the early initiation of synaptic scaling (Turrigiano (2008), Becker et al. (2013)). TNFα is also involved in synaptic scaling in neuromuscular junction (Knogler et al. (2010)).

1.2.3.3 Arc

Arc expression levels in a neurone is highly regulated by neuronal activity (Rao et al. (2006)) and has clear implications in formation of memory (McIntyre et al. (2005), Guzowski et al. (2000)). It has also been shown that disrupting the level of Arc by over expression and knock out strategies can disrupt homeostatic synaptic scaling (Shepherd et al. (2006)). It has also been shown that Arc is involved in AMPA receptor
trafficking together with endophilin and dynamin (Chowdhury et al. (2006)). Arc has also been linked with endocytosis of GluR1 (Chowdhury et al. (2006)) and GluR2/3 (Rial Verde et al. (2006)). The induction of Arc is also specifically down stream to ERK1/2 dependent transcriptional pathway but not mTOR dependent transcriptional pathway (Bateup et al. (2013)). It has also recent been revealed that Arc is relocated to the nucleus in response to activity to alter GluR1 levels (Korb et al. (2013)). This suggests that Arc is involved in globally alteration of synaptic strength rather than local alterations.

1.2.3.4 Ca\(^{2+}\) related molecules

The initial model of homeostatic plasticity predicted Ca\(^{2+}\) as an internal sensor of activity (LeMasson et al. (1993)). If this model is true, it would mean that the molecular machinery used for homeostatic plasticity would be initiated by the change in the intracellular Ca\(^{2+}\). Blocking L-type Ca\(^{2+}\) channel mimics the effect of AMPA receptor blocking by increasing GluR1 levels (Thiagarajan et al. (2002)) and GluR2 levels (Ibata et al. (2008)) and also results in changes in mEPSC properties of the cell. Also an interesting observation is local application of NMDA receptor antagonist but not AMPA receptor antagonist during activity block induces fast local homeostatic plasticity. As NMDA receptor is Ca\(^{2+}\) permeable and majority of AMPA receptor subtypes are not, these result indicates that the homeostatic mechanisms caused by AMPA blockage is due to Ca\(^{2+}\).

So what would be the down stream molecules that senses the Ca\(^{2+}\) to have physiological impact? Thiagarajan et al. (2002) showed that the two subtypes of calcium calmoduline dependent protein kinase 2 (CAMK2) are regulated by neuronal activity. Interestingly the two subtypes (CAMK2α and CAMK2β) were regulated inversely. Activity induced potentiation of CAMK2α and induced down regulation of CAMK2β. CAMK2α transfection significantly lowered the frequency of mEPSCs and CAMK2β significantly increased the frequency of mEPSC suggesting opposite effects of the two subtypes. However only CAMK2α increased the mEPSC amplitude whereas CAMKβ did not induce any change in the amplitude (Thiagarajan et al. (2002)). This subtype specific actions are shown to be acting through guanylate kinase associated protein (GKAP) phosphorylation at different sites causing bidirectional effects in synaptic proteins (Shin et al. (2012)). CAMK2 was recently shown also to be involved in scaling inhibitory synapses through gephyrin phosphorylation (Flores et al. (2015)).

As the blockers of CAMK2 used in Thiagarajan et al. (2002) also blocks calcium/calmoduline dependent protein kinase 4 (CAMK4) Ibata et al. (2008) tested the involvement of CAMK4 in homeostatic plasticity. The study revealed that the nuclear
localisation of CAMK4 is blocked in response to TTX and also the dominant negative form of CAMK4 increased mEPSC amplitude. CAMK4 and its activator CAMKK was required for synaptic depression in response to increased activity (Goold and Nicoll (2010)).

1.2.3.5 β3 integrin

Homeostatic synaptic plasticity has been linked with synaptic morphological changes (Seeburg and Sheng (2008), Goold and Nicoll (2010)). Therefore it has been hypothesised that molecules involved in building the pre and the post synapses would be involved in homeostatic plasticity. Integrins are one of synaptic adhesion molecule which are involved in binding of pre and post synapses (Yamagata et al. (2003)). Expression of β3 integrin induced scaling up of mEPSCs through regulating GluR2 surface expression (Cingolani et al. (2008)).

1.2.3.6 Major histocompatibility complex (MHC) class I

MHC class1s are primarily involved in immune reactions but are also shown to be expressed in an activity dependent manner in CNS neurones (Corriveau et al. (1998)). Neurones with reduced levels of MHC class1 are shown to have increased mEPSC frequency. The neurones also had increased number of vesicles at the presynaptic terminal but did not show any changes in the postsynaptic terminal (Goddard et al. (2007)). Increased vesicle number is shown to increase release probability hence increasing the activity of the presynaptic neurone (Murthy et al. (2001)). As the presynaptic vesicle release is detected in the postsynaptic neurone as a mEPSC event, this is detected in the post synaptic neurone as increased mEPSC frequency.

1.2.3.7 Nitric oxide synthase

Nitric oxidase synthase is coupled to glutamatergic signalling in hippocampal neurones (Garthwaite (2008)). Steinert et al. (2011) showed that nitric oxide (NO) signalling is involved in modulating the subunit composition of voltage gated potassium channels in a phosphorylation dependent manner. This change in subunit composition, namely reduction of Kv3 and enhancement of Kv2 in response to activity, impacts the action potential repolarisation in a homeostatic manner in response to glutamatergic synaptic activity. Recently it has been shown that NO signalling impacts translation rates of VGAT (vesicular GABA transporter) indicating a role of nitric oxide synthase in inhibitory synapses (Peterson et al. (2015)).
1.2.3.8  Polo like kinase 2 (Plk2)

Plk2 is a serine/threonine kinase which is also essential for down scaling of synapses in response to enhanced activity (Seeburg et al. (2008)). Plk2 is also involved in change in synaptic and intrinsic property changes in response to epileptiform activity through the mTOR signalling pathway which is involved in synaptic protein synthesis (Seeburg and Sheng (2008), Sun et al. (2013)). Downstream targets of this have been suggested to be Ras and Rap, signalling molecules also involved in long term potentiation (LTP) and long term depression (LTD) (Lee et al. (2011)).

Pumilio 2 was discovered to change translation of sodium channels in response to activity to homeostatically change intrinsic properties of the neurone (Driscoll et al. (2013)). Pumilio 2 has also been shown to be essential in synaptic depression and targets Plk2 (Fiore et al. (2014)).

1.2.3.9  Retinoic acid

all-trans retinoic acid (RA) increases synaptic strength in primary hippocampal culture. This is also dependent on protein synthesis (Aoto et al. (2008)). The expression of RA is triggered by the depletion of synaptic Ca^{2+} concentration (Wang et al. (2011)) thus homeostatically upregulating the synapic activity in response to decrease in activity. RA also decreases the strength of inhibitory synapses indicating coordinated action in both excitatory and inhibitory synapses (Sarti et al. (2013)).

1.2.3.10  Methyl-CpG binding protein 2 (MeCP2)

Mutations in MeCP2 is a cause of Rett syndrome, a leading cause of intellectual disability in females (Chahrour and Zoghbi (2007)). Blackman et al. (2012) showed that a mouse model of Rett syndrome which has mutated MeCP2 shows disrupted homeostatic synaptic plasticity. Bicucullin induced scaling down of synapses was shown to involve phosphorylation and increase in the level of MeCP2, which then regulates the transcription of GluR2 (Zhong et al. (2012), Qiu et al. (2012)).

1.2.3.11  Other molecules

In addition to molecules discussed above there are many other molecules which are being linked to homeostatic plasticity. Here I will briefly discuss some of the molecules.

Endoplasmic reticulum has been linked with intracellular calcium levels and local protein synthesis. There has been evidences that there is a remodelling of spine appa-
ratus, which is composed of stacked endoplasmic reticulum, in response to change in activity (Vlachos et al. (2013), Maggio and Vlachos (2014)). Phosphatidylinositol 3,5-bisphosphate has also been linked to AMPA receptor trafficking (McCartney et al. (2014)). Rab3-GAP inhibits homeostatic presynaptic plasticity by affecting the vesicle release (Müller et al. (2011), Muller et al. (2012), Müller et al. (2015)). EphA4 also downregulates activity mediated GluR1 through anaphase-promoting complex which degrades GluR1 (Fu et al. (2011)). GRIP1, an AMPA receptor binding protein, is also essential in scaling up of synapses in response to depletion of activity (Gainey et al. (2015)). Narp, an activity dependent early gene, has been linked to scaling of inhibitory synapses (Chang et al. (2010)).

These vast amount of molecules linked with neuronal homeostasis suggests that homeostatic plasticity is likely to involve changes in multiple molecules affecting multiple cellular pathways to achieve homeostasis.

1.2.4 Conclusion

Work above shows complex nature of homeostatic plasticity in terms of its physiological mechanism and molecular mechanism. How a neurone adapts homeostatically to suppression or enhancement of activity may depend on many factors such as its cell types and the developmental stage. Also there is a shortage of studies assessing the physiological and molecular mechanisms in vivo with most studies focusing on culture systems.

1.3 Aim and organisation of the thesis

As disruption of homeostatic plasticity is involved in many different form of neurological diseases such as fragile X syndrome and Rett syndrome (Sarti et al. (2013), Blackman et al. (2012), Ramocki and Zoghbi (2008)), it would be important to understand the molecular pathway involved in homeostatic plasticity to potentially use as drug targets for neurological diseases. In vitro system has advantages in the experimental ease but the cellular environment is dramatically different from its natural environment. This may or may not cause different mechanism of adaptation from in vivo. Therefore it would be ideal to investigate the molecular changes in an in vivo system where neurones are in its natural environment.

The project will aim to investigate molecular mechanisms involved in adaptation to decreased neuronal excitability in vivo with 3 specific aims. I chose to manipulate
neuronal activity in vivo for my experiments due to the reasons above. Additionally, pharmacological manipulations of activity using TTX (for an example of such study in vivo see Echegoyen et al. (2007)) has no cell type specificity. As neurones exist in a network, blocking activity in all cells would mean pre and post synaptic mechanism cannot be easily dissected out. To overcome these problems in cell type specificity I have decided to perturb neuronal activity by genetically manipulating a subtype of neurones, excitatory granule cells in the DG and pyramidal cells in CA1.

The hippocampus is involved in memory formation and much synaptic plasticity work has focused on this structure (Neves et al. (2008)). Also homeostatic plasticity is observed in hippocampal neurones in cultures, slice cultures and in vivo (Thiagarajan et al. (2005), Burrone et al. (2002), Vlachos et al. (2013), Echegoyen et al. (2007)). As the hippocampal activity is such important part of formation of various types of memory (Ramirez et al. (2013), Liu et al. (2012), Ramirez et al. (2015)) homeostatic mechanism in the hippocampus would be of biological importance also. The fact that the hippocampus has defined regions where the soma of single subtype of neurones exist at high densities also makes it convenient for experiments.

First aim: Develop and characterise lentivirus and AAV system to modify neuronal gene expression in vivo

The manipulation I have chosen to alter neuronal activity by genetically over express Kir2.1 with mCherry tag. Overexpression of Kir2.1 lowers membrane conductance and resting membrane potential thus suppressing activity in neurones (Burrone et al. (2002), Hou and Zhang (2008), White et al. (2011)). Also as the channel is tagged with mCherry, this allows visualisation of expression levels histologically and in flow cytometric analysis. My first aim in this project is to develop and characterise virus systems to alter neuronal genetic expression at specific regions in vivo and to characterise the anatomical changes to ensure no adverse effects have been caused by the manipulation. The work concerning the first aim will be presented in chapter 3 under the title "Anatomical analysis of Kir2.1 expression with lentivirus and AAV".

Second aim: Characterisation of physiological adaptation after Kir2.1 overexpression

After the expression of Kir2.1 in neurones it is important to confirm the changes caused by the Kir2.1 and subsequent adaptation of neurones over expressing Kir2.1. To do this I have chose to use slice patch clamp methods to measure various electrophysiological properties. In vivo patch clamp of Kir2.1 expressing cells would reveal the homeostatic mechanism at its most natural state but this is extremely difficult tech-
nique to perform with low yield of data (Tao et al. (2015)). Using slice patch clamp techniques and nearby cells as control cells I aimed to investigate the mechanism and the nature of adaptation in granule cells and pyramidal cells. Work concerning the physiological mechanism of adaptation will be presented in chapter 4 under the title "electrophysiological analysis of cell properties after Kir2.1 overexpression".

Third aim: Characterisation of molecular mechanism responsible for the adaptation after Kir2.1 over expression

Previous studies looking at molecular mechanisms used a targeted approach using a defined target and manipulating the target to reveal the involvement of the molecule in homeostatic process. This approach has resulted in many molecular candidates as discussed above. Another way to study this is to use a un-biased approach and investigate the whole transcriptome during adaptation. I reasoned that as Kir2.1 is overexpressed in neurones at the time of physiological and genetical analysis (2 weeks after introduction of viral vectors), the molecular mechanism responsible for the adaptation would also be active at the time of analysis. The analysis of gene expression was targeted to a specific neuronal type. It is well appreciated that gene expression profiles of different neuronal subtypes are different (Sugino et al. (2006)). Taking the whole hippocampus as experimental sample may increase background variability which may mask the important changes responsible for the adaptation. Also as the virus did not infect every cell in the region of interest uninfected cells would also increase background variability. To overcome this, I aimed to isolate the pure population of virus infected cell type of interest by using Fluorescence activated cell sorting (FACs) method (White et al. (2011)). FACs isolated pure cell population was used to isolate the RNA for RNAseq and differential gene expression analysis. Work done to develop FACs method and the subsequent RNAseq is presented in chapter 5 under the title "molecular changes after Kir2.1mCherry expression".

In chapter 2 I will describe the general experimental protocols I have used but the detailed design of each experiments will be presented with the results in chapter 3, chapter 4 and chapter 5.
2.1 INTRODUCTION

To investigate molecular changes after induction of homeostatic plasticity \textit{in vivo}, I first genetically alter neuronal function with virus mediated gene delivery. Second, I confirmed the physiological changes of the virus infected cells with slice patch clamp method and third, I analysed the genetic changes after the manipulation using RNA sequencing. This approach provides a powerful strategy to manipulate neuronal activity \textit{in vivo} in a cell type specific manner and subsequently access the physiological and genetic changes after the manipulation. Implementation of this strategy requires an experimental methodologies involving molecular biological techniques, flow cytometry and electrophysiology.

The general experimental methods for this study will be described in this chapter. Specific methods and experimental designs relevant for specific chapters will be described in the chapter under methods section.

All animal experiments were done according to guidelines provided by the University of Edinburgh’s Animal Welfare Committee and UK Animal (Scientific Procedures) Act 1986. All the experimental protocols involving use of animals were approved by the University of Edinburgh Biological services.

2.2 GENETICALLY ALTERING NEURONAL FUNCTION \textit{in vivo}

2.2.1 Generation of lentivirus

All the DNA constructs used for lentiviral study were made by Dr. Melanie White and the details of the synthesis are described in White et al. (2011). The viral constructs were grown in Stbl 3/TOP10 chemically competent cells (Invitrogen) and harvested with Hispeed Plasmid Maxi Kit (Qiagen). Lentivirus was produced as described in White et al. (2011). In Brief, lentiviral plasmid and helper plasmids (psPAX2 and pVSVG, courtesy of Didier Trono, EPFL, Lausanne, obtained via Addgene and Invitrogen respectively) were transfected into HEK293FT cells (Invitrogen) with calcium
2.2 GENETICALLY ALTERING NEURONAL FUNCTION in vivo

phosphate. The cells were then incubated in serum free media for 3 days and the virus was concentrated in centricon Plus-80 filter device (Millipore). The functional titre was calculated by counting the mCherry fluorescent cells after lentivirus infection with serial dilution of the virus. The components used in this study are summarised in table 2.1.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Promoter</th>
<th>Transgene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir2.1-mCherry</td>
<td>CAMKII(0.4)</td>
<td>Kir2.1-mCherry</td>
</tr>
<tr>
<td>mCherry</td>
<td>CAMKII(0.4)</td>
<td>mCherry</td>
</tr>
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</table>

Table 2.1: List of lentiviral plasmid used to generate lentivirus

2.2.2 Generation of AAV

For generation of AAV plasmids I used gateway cloning method provided by Invitrogen. All the entry vector and the destination vector were from the molecular toolbox developed by White et al. (2011). Kir2.1-mCherry or mCherry along with CAMKII(0.4) promoter and oPRE sequence were ligated into the AAV destination vector with LR clonase 2 plus enzyme. The reaction was kept at room temperature (15 - 20°C) overnight. 3µl of the reaction was mixed with 25µl of STBL3 cells (Invitrogen) and incubated on ice for 30 minutes before incubation at 45°C for 45 seconds. The cells were incubated on ice for 2 minutes and incubated at 30°C for 90 minutes on a shaker after addition of 200µl of S.O.C. media (Invitrogen). The reaction was then plated out on LB agar plate containing 50µg/ml ampicillin and incubated at 30°C overnight. The resulting colonies were collected and grown in LB medium (Sigma) containing 100µg/ml ampicillin at 30°C at least for 7 hours. The plasmid was then purified with QIAprep spin mini prep kit (Qiagen) according to the manufacturer’s protocol. The plasmid was screened for false positives using restriction digests for each plasmid and the true positive cultures were grown in a larger scale and the plasmid from the large scale culture was purified with Hispeed Plasmid Maxi Kit (Qiagen). Digests used in this study are presented in table 2.2. The plasmid was then transfected into HEK293FT cells using FuGENE6 (Roche) and mCherry expression was screened for validation of functionality. Concentration of the plasmids were measured on nanodrop 1000 or nanodrop 2000 (Thermo scientific).

Validated plasmids were used to make AAV. Detailed procedures for AAV generation and purification are described in McClure et al. (2011). AAV used for this study were generated by both myself and Dr. Christina Mclure. In brief, HEK293
cells were transfected with the AAV construct and helper plasmids (pRVL, pH21 and pFdelta6) with calcium chloride at 37°C overnight with 5% CO₂. The virus was purified with heparin column purification 72 hours after transfection. Purified virus was resuspended in sterile PBS.

### 2.2.3 Stereotaxic injection into hippocampal regions

To deliver the viruses into a precise location in the brain, stereotaxic surgery was used. Stereotaxic surgery allows delivery of small volumes of viruses into a small region of the brain. This means that the manipulation can be targeted to a specific region rather than globally changing the gene expression. This also allows genetic manipulation in the adult brain, by-passing the developmental involvement of the gene. The same procedure was followed for all surgeries performed for histological, electrophysiological and genetic profiling work presented in this study. For all injections, 4-6 weeks old mice were used. All experimental procedures were done 2 weeks after the surgery unless stated otherwise.

![Figure 2.1: Schematics of mouse skull reference points. Bregma and lambda are shown and all coordinates were measured from bregma. Figure adapted from Cetin et al. (2006)](image.png)

**Table 2.2: List of AAV plasmid digest**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Digest</th>
<th>Resulting band size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir2.1-mCherry</td>
<td>Ndel/KpnI</td>
<td>4654/2959</td>
</tr>
<tr>
<td>mCherry</td>
<td>Ndel/KpnI</td>
<td>6118</td>
</tr>
</tbody>
</table>

C57BL6 or YFP-H mice (development in Feng et al. (2000)) were anaesthetised with isoflurane and the head was shaven to clear the incision area. The mouse was
2.2 Genetically Altering Neuronal Function in vivo

<table>
<thead>
<tr>
<th>Target region</th>
<th>A/P</th>
<th>M/L</th>
<th>D/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>DG</td>
<td>-2.0</td>
<td>±1.3</td>
<td>-1.8</td>
</tr>
<tr>
<td>CA1</td>
<td>-2.0</td>
<td>±1.6</td>
<td>-1.4</td>
</tr>
</tbody>
</table>

Table 2.3: Coordinates used for stereotaxic surgery. A/P is anterior/posterior, M/L is medial/lateral and D/V is dorsal/ventral. All the coordinates were measured from bregma.

<table>
<thead>
<tr>
<th>Target region</th>
<th>A/P</th>
<th>M/L</th>
<th>D/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal anterior</td>
<td>-1.6</td>
<td>±1.1</td>
<td>-1.6</td>
</tr>
<tr>
<td>Dorsal mid</td>
<td>-2.2</td>
<td>±2.2</td>
<td>-1.6</td>
</tr>
<tr>
<td>Dorsal posterior</td>
<td>-3</td>
<td>±3</td>
<td>-4.5</td>
</tr>
<tr>
<td>Mid posterior</td>
<td>-3</td>
<td>±3</td>
<td>-3</td>
</tr>
<tr>
<td>Ventral posterior</td>
<td>-3</td>
<td>±3</td>
<td>-2.2</td>
</tr>
</tbody>
</table>

Table 2.4: Coordinates used for stereotaxic lesion surgery. A/P is anterior/posterior, M/L is medial/lateral and D/V is dorsal/ventral. All the coordinates were measured from bregma.

mounted on a stereotaxic surgery frame and 0.3ml of 0.3mg/ml vetcergesic was given via subcutaneous injection. Tear drops were applied to prevent drying of the eyes during the surgery. The incision area were cleaned with videne antiseptic solution (Adams) and the incision was made to expose the lambda and bregma of the skull. The head was adjusted so that the lambda and bregma were at level. A small pin hole was made in the skull at the injection site (measured from bregma, see figure 2.1) with a dremel drill with 0.5 - 0.7 mm diameter tip. 500nl of virus was delivered at 100nl/min rate with pre-calibrated pipettes with a tip diameter of 6-10 µm. The pipette was left in place for 5-10 minutes before being slowly retracted to minimise back flow. The wound was closed with Vetbond and the mice were supplied with analgesic jelly for post operative pain. 0.2ml of warm saline were subcutaneously injected into the mice (in two 0.1ml injections) to prevent dehydration. Mice was kept on 37$^\circ$C heating mat throughout the whole procedure.

DG and CA1 of the hippocampus were targeted for this study and the coordinates used for stereotaxic surgery are given in table 2.3.

The hippocampal lesion was performed by Dr. Helen Ramsden. She followed the same preparation steps for stereotaxic surgery as described above and injected 100nl of ibotenic acid (2.5mg/ml) at each coordinated given in table 2.4 to lesion the whole
2.2 Genetically altering neuronal function in vivo

The animals were returned to their home cage after surgery for at least 1 week and were scarified for downstream experiments. Their health was monitored 24 hours after surgery, 48 hours after surgery and regularly until they were scarified. The experiment was terminated if any distress signs are detected in the mice post surgery before the sacrifice.

2.2.4 Histological studies

For histological examination of the brain after stereotaxic injection, the brain was perfused with 4% paraformaldehyde (PFA). The mice were first scarified by overdose of sodium pentobarbital. A lateral incision was made in the abdominal area to expose the diaphragm and the diaphragm was pierced. The rib cage was removed to expose the heart. A perfusion needle was inserted into the left ventricle of the mouse and phosphate buffered saline (PBS) was pumped into the heart for 4 mins while multiple incisions were made on the liver to drain the blood and excess PBS. Ice cold 4% PFA was perfused for 10 mins. The brain was obtained and was incubated in 4% PFA overnight at 4°C for post fixation. The fixed brain was then washed in PBS to remove the residual PFA and was transferred to 30% sucrose solution until fully submerged.

Free floating sections were made on a freezing microtome (HM450 and KS34 Thermo Scientific). Before sectioning, the cerebellum was removed in the guide to ensure that the brain is level with the blade. This ensures that the hippocampal area from the two hemispheres in a section are on the same coronal plane. The brain was frozen at -25°C. The whole hippocampal area was collected as shown in figure 2.2 in 40 µm sections. The sections were kept in PBS until further processing.

<table>
<thead>
<tr>
<th>Staining</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuN</td>
<td>Anti-NeuN (Millipore)</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td>mCherry</td>
<td>DsRed (Clontech)</td>
<td>Alexa Fluor 568</td>
</tr>
<tr>
<td>Nissel staining</td>
<td>None</td>
<td>Neurotrace (Invitrogen)</td>
</tr>
</tbody>
</table>

Table 2.5: List of antibodies used for histological studies.

For antibody staining, the sections were permeabilised with 0.5% triton X 100 (TX100) in PBS for 30 minutes. The section were then blocked in 1.5% Normal goat serum (NGS) in PBS for at least 1 hour. The primary antibody was applied to the
Figure 2.2: Sectioning methods. (A) The cerebellum was cut as shown by the black line. (B) 40 µm sections were collected as shown. Example of start, mid and end point of collection are shown. Left panel shows relevant area in Brain explorer tool from Allen Brain Institute. Right hand panel shows respective example photos from perfused brain. Black arrows denote the hippocampal region.

Sections over night at 4°C at appropriate dilution in 1.5% NGS in PBS. The primary antibody was washed off 3 times in PBS for 5 minutes each and the desired secondary antibody (1:500 in 1.5% normal goat serum (NGS) in PBS) was applied for 1
hour at room temperature. The secondary antibody was washed off 3 times with PBS, 5 minutes each and the sections were mounted on a glass slide and cover slipped in mowiol mounting media (96 mg/ml Mowiol (Sigma), 240 mg/ml Glycerol (Sigma), 25 mg/ml DABCO (Sigma), 0.1M TrisHCl (Sigma)).

For hematoxylin and eosin staining and fluoro Jade C (FJC) staining (Invitrogen), the sections were mounted on SuperFrost Plus slides (Thermo Scientific) and allowed to dry before the staining process. All staining was done according to manufacture’s instructions.

After electrophysiological studies, biocytin filled cells were counterstained for morphological analysis. After the recording was completely the recording pipette was slowly retracted to minimise disruption of the tissue. The slice was then incubated in 4% PFA solution in 4°C for at least overnight and for a maximum of 1 week. The PFA was then washed with PBS for 5 minutes 3 times. The slice was permeablised with 0.5% TX100 in PBS for 3 hours and then blocked with 1.5% NGS in PBS with 0.3% TX100 for 2 hours. Blocked slices were incubated in DsRed antibody solution (1:400 in 1.5% NGS in PBS with 0.3% TX100) overnight at room temperature. DsRed antibody solution was washed with PBS with 0.3% TX100 5 times each for 10 minutes. The slices were then incubated with alexa594 rabbit secondary antibody (invitrogen, 1:500 dilution) and streptavidin-alexa488 (Invitrogen, 1:1000) in 1.5% NGS in PBS with 0.3% TX100 for 24 hours at room temperature. The slice was then washed with PBS with 0.3% TX100 5 times each for 10 minutes and mounted and coverslipped with vectashield mounting medium (Vector lab).

All imaging was done on on Nikon A1R FILM confocal microscope, Zeiss LSM510 confocal microscope and Leica TCS-NT. The images were processed with image j.

### 2.3 Electrophysiological Methods

To investigate the changes in electrophysiological properties after Kir2.1 expression in vivo I used virus mediated gene manipulation and stereotaxic surgery for virus delivery as described above.
2.3.1 Slice preparation

I euthanised the mice by cervical dislocation without any anaesthetisation. The brain was then carefully removed and transferred to ice cold cutting artificial cerebrospinal fluid (aCSF) (86 mM NaCl, 1.2 mM NaH₂PO₄, 2.5 mM KCl, 25 mM NaHCO₃, 25 mM glucose, 75 mM sucrose, 0.5 mM CaCl₂, and 7 mM MgCl₂) saturated with carbogen (95% O₂ and 5% CO₂) for at least 2 minutes to ensure complete cooling of the tissue. The brain was gently removed from the solution with a spatula and placed on a cutting aCSF moistened filter paper. The rostral third of the cortex was removed at a 10°- 30° angle and the cerebellum was also removed with a razor blade. The brain was partially hemisected leaving the rostral end intact. The brain was mounted, rostral end facing down, on the stage of the vibrotome with superglue. The brain was then completely submerged in ice cold cutting aCSF saturated with carbogen and was constantly supplied with carbogen by bubbling the solution throughout the slicing process. 400µm slices were obtained at cutting speed of 0.6mm/second with the blade at 20°. Slices containing hippocampus region were collected in standard aCSF. Collected slices were incubated in 37°C standard aCSF for 15 minutes and cooled to room temperature for at least 50 minutes before recording at 33 to 37°C with constant supply of carbogen. The standard aCSF had the following composition: 124 mM NaCl, 1.2 mM NaH₂PO₄, 2.5 mM KCl, 25 mM NaHCO₃, 20 mM glucose, 2 mM CaCl₂, and 1 mM MgCl₂.

2.3.2 Whole cell recordings

The slice was viewed under 4X objective lens on an upright Olympus BX51 WI microscope (Olympus UK Ltd.) fitted with Normarski differential interference contrast imaging (DIC). Images were acquired with infrared-capable mono 12 bit CCD camera (Q-/Imaging) and Q-capture software. A general region of interest, DG or CA1, was identified based on the tightly packed cell body layer. Viability of cells and the fluorescent profile of the cells were determined with a 40X water immersion objective lens. For fluorescence detection, DIC prisms were removed from the microscope and the slice was excited with X-Cite 120Q Arc lamp (Excelitas Technologies) with Chroma 49008 ET-mCh/tR emission filter.

To record the membrane voltage or the membrane current I used the whole cell patch clamp method (Neher and Sakmann (1976), Neher et al. (1978), Hamill et al. (1981)). All the recordings were obtained at 33 to 37°C temperature with glass pipette.
with 4 - 8 MΩ tip resistance pulled with a P-97 Flaming/brown micropipette puller (Sutter instruments). Glass pipettes were filled with K gluconate filling solution or Cs gluconate filling solution. K gluconate solution contained in mM: 120 K gluconate, 10 NaCl, 2 MgCl2, 0.5 K2EGTA, 10 Hepes, 4 Na-ATP and 0.3 Na-GTP, pH 7.2, biocytin. Cs gluconate solution contained in mM: 100 gluconic acid, 0.2 EGTA, 5 MgCl2, 40 Hepes, 2 Na-ATP, 0.3 NA-GTP with 5 QX314, 1 BaCl2, 10 TEA and biocytin. The silver electrode was chlorides by holding it under + 9V in 9% NaCl solution. A tight seal with a seal resistance of >1GΩ was made between the patch pipette and the cell. After a stable seal has formed, the cell membrane was ruptured by applying a slight negative pressure to enter the whole cell configuration. For simultaneous paired patching experiments a tight seal was achieved on one cell and the same steps were taken for the other cell. To minimise difference in electrical property caused by plasticity during whole cell configuration, both cells were first stably sealed before rupturing simultaneously.

For current clamp experiments pipette capacitance was neutralised by amplifier estimated values and fine tuned manually. Bridge balance was applied to compensate for series resistance. The access resistance value measured was always less than 30 MΩ. For voltage clamp pipette capacitance, membrane capacitance and series resistance compensation settings were adjusted until the fast and slow transients of the current response to -10 mV voltage step were removed. Series resistance was compensated at 70% for all experiments. Current clamp recordings were made using Multi-clamp or AxoClamp amplifiers (Molecular Devices) and voltage clamp recordings were made using a Multiclamp amplifier (Molecular Devices). Data were sampled at 20 kHz and digitised using a Digidata 1320A (MolecularDevices). The drugs were either applied to the recording aCSF and perfused into the recording chamber or mixed with the intracellular solution to specifically affect the recorded cell.

Stimulating electrodes were made by applying silver paint to the surface of a normal aCSF filled recording pipette. The silver paint was not applied to the very end of the electrode to avoid contact with silver and the tissue but was always in contact with the aCSF in the recording chamber. To improve the delivery of the stimulus, the tip of the pipette used to make the stimulating electrodes were widened by changing the configurations on pipette puller. The positive end of the stimulator was inserted into the stimulating electrode and the negative end of the stimulator was wrapped around the silver applied surface of the stimulating electrode. The intensity of the stimulation was controlled manually with analogue dials on the stimulator while the timing was controlled digitally through the digitiser.
To investigate the molecular changes after Kir2.1-mCherry expression, I have used FACs to isolate the pure population of Kir2.1-mCherry expressing cells and RNA-seq to analyse the transcriptome of the Kir2.1-mCherry expressing cells. First I will describe experimental procedure regarding the FACs of Kir2.1-mCherry expressing cells and describe the procedure for RNA-seq.

2.4.1 Cell dissociate preparation

For expression profiling experiments it is important to eliminate the possibility of RNase contamination in the solutions used for the procedure. RNase contamination will result in degradation of sample RNA which may reduce the quality of the data obtained from the sample. I therefore added 0.1% diethylpyrocarbonate (DEPC) to water used to make up the solutions. The water was incubated at room temperature overnight with 0.1% DEPC and DEPC was inactivated by autoclaving. PBS used for this study was also treated with DEPC. The protocols described here were based on the FACs sorting protocol described in White et al. (2011).

Acute 400µm slices were prepared as described before, but in DEPC treated cutting aCSF. The slices were incubated in 30° C cutting aCSF for at least 10 minutes. The slices were then transferred to micro dissecting plate and the region of interest was dissected under a light dissecting microscope. The dissected region was transferred to 900µl of cutting aCSF supplemented with 100µl of papain (200U/ml, Sigma-Aldrich) for 45 minutes in 37° C with 5% CO₂. Papain activity was stopped by adding 100 µl bovine serum albumin (BSA) (10 mg/ml; Sigma-Aldrich), 11µl fetal calf serum (FCS) (Invitrogen), 10µl DNase (1,000 U/ml, Sigma-Aldrich), and 10µl leupeptin (5mM, Sigma-Aldrich). A single cell suspension was prepared by passing the papain treated tissue through a fire polished glass pipette until no clumps are visible. The suspended tissue was spun for 5 minutes at 800 rpm. The aCSF was removed and the samples were re-suspended in FACs buffer (1% FCS in PBS) typically in 500µl per single hemisphere. Dissociated samples were kept on ice until the sorting experiment which was typically 30 minutes after the sample preparation.
2.4.2 FACs method and RNA isolation

Before sorting, the samples were filtered through the 70μm cell strainer. DAPI (Life Tech, final concentration 1μg/ml) was added to the filtered sample. DAPI enters cells with compromised membrane to bind to the nucleus causing fluorescence. As compromised membrane is an indicative of dying cells, DAPI positive cells were excluded from the sort. DAPI added samples were then analysed on FACs for size, granularity and fluorescence. Detailed procedure of sorting gate determination is presented in figure 2.3.

![Sorting gates for cellular particles](image)

Figure 2.3: Sorting gates for cellular particles. (A) From all the particles in the cell dissociate, only the singlets were selected by selecting the particles with liner relationship between the values of forward scatter (FSC) area and height. (B) From the singlet population the cellular particle population was selected based on the size (measured by FSC-A) and granularity (measured by side scatter A (SSC-A)). (C-D) From the cellular particle population the live cell population was selected based on the DAPI fluorescence. (C) shows DAPI fluorescence before adding DAPI and (D) shows DAPI fluorescence after adding DAPI in the same sample. The numbers on each panel indicates percentage of parent population.

The cell dissociate was initially analysed for the the area of the forward scatter signal (FSC-A) and the height of the forward scatter signal (FCS-H). Particles with same values indicate a round object, likely to be a single cell. However particles with different values for FCS-A and FCS-H are likely to be two particles bound together.
As these can be a source of contamination they were removed from the sort (figure 2.3 A). Generally 80% of the cell dissociate were singlets. From the singlets the cellular particles were determined. Firstly all the particles were plotted with FCS-A and side scatter area (SSC-A) value. There were 3 populations with different sizes (FCS-A) and a wide range of granularity (SSC-A). The largest population was selected as the smaller populations were mostly dead cells (figure 2.3 B). The cellular particles were analysed for their DAPI signal. DAPI signal was detected with V450 50-A laser. Figure 2.3 C shows DAPI signal from cellular particles before the addition of DAPI. There were a few particles with DAPI autofluorescence. The large DAPI negative population was selected as live population. Addition of DAPI resulted in DAPI labelling of membrane compromised dead cells. From this live cell population the fluorescence of the fluorophores were analysed and the desired populations were sorted.

The cells were sorted into 100µl of the RNA extraction buffer from the picopure RNA isolation kit (Arcturus, life technologies). RNA was isolated according to the manufacturers protocol with on column DNase treatment with RNase-Free DNase Set (Qiagen). Purified RNA samples were kept at -75°C until the downstream experiments.

2.4.3 RNA sample quality check and processing

The quality of the RNA as well as the purity of the sorts were analysed by investigating the resulting RNA sample. For RNA quality and quantity assessment I used a Bioanalyzer 2100 (Agilent) with a RNA 6000 Pico Kit (Agilent) according to the manufacturer’s protocol. For testing the purity of the sample, I used qPCR to determine the levels of cell markers in the RNA sample. For qPCR I used TaqMan® RNA-to-C₅™ 1 step kit (Applied Biosystems) with TaqMan® gene expression assays (Life Technologies). The full list of gene expression assays used in this study is shown in table 2.6. Reference RNA from the whole hippocampus and DG were obtained by obtaining acute slices as above. Either the whole hippocampus or DG were microdissected and treated with papain and dissociated. The dissociated tissue was centrifuged for 5 minutes at 800rpm and the supernatant was removed. The pelleted tissue was resuspended in 100µl of RNA extraction buffer and the RNA was isolated with picopure RNA isolation kit with on column DNase treatment. The concentration and temperature cycle were according to the manufacturer’s protocol and each reaction was made up to 20 µl with DEPC treated water. Standard curves for each gene expression assay were calculated with reference RNA from the whole hippocampus. 5 serial dilutions
were used for the standard curve. Ct (cycle threshold) results from the qPCR were converted to RNA concentration by comparing the value with the standard curve of the assay which was performed prior to the experiment. All gene expression within the sample was normalised to the level of housekeeping gene (HPRT) then normalised to the levels measured from non-sorted sample from the region of interest.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>Mm01545399_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>MCM6</td>
<td>Mm00484848_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>NeuroD</td>
<td>Mm01326464_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>GFAP</td>
<td>Mm01253033_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>GAD2</td>
<td>Mm00484623_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>NOS1</td>
<td>Mm00435175_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>RGS17</td>
<td>Mm00490876_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>ACVR1C</td>
<td>Mm03023957_m1</td>
<td>FAM</td>
</tr>
<tr>
<td>KCNQ3</td>
<td>Mm00548884_m1</td>
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</tr>
<tr>
<td>MAL</td>
<td>Mm01339780_m1</td>
<td>FAM</td>
</tr>
</tbody>
</table>

Table 2.6: List of qPCR primer used for this study. Details of the primer sequences and the target transcripts are available on Life Technologies website.

Satisfactory RNA samples were spiked with ERCC RNA Spike-In Mix (Ambion, Life Technologies) (0.5µl of 1:500,000 dilution). RNA and RNA spike in mix was then amplified with Ovation® RNA-Seq System V2 Kit (Nugen) according to the manufacturer’s protocol. The resulting cDNA was quantified with NanoDrop (Thermo Scientific).

2.4.4 Library preparation and RNAseq

Amplified cDNA was used for library preparation for RNAseq. The library prep, sequencing and mapping was performed by Edinburgh Genomics facility (University of Edinburgh) using TruSeq Total RNA-seq kit. The sequencing run generated 36 million to 50 million reads per sample for DG granule cell experiments. Sequencing was performed on the Illumina MiSeq and raw data processed with CASAVA 1.8. Reads were mapped to the mouse genome (Mus_musculus.GRCm37_release74 from ensembl) using tophat version 2.07 with the parameters "-r 40 -mate-std-dev 22 -solexa1.3-quals". Duplicates were removed using samtools version 0.1.18. Reads mapping to genes were counted using htseq-count version 0.5.4p5 with the parame-
-s no (reads not stranded). The scripts are presented in the appendix.
3.1 INTRODUCTION

There are number of ways in which one can experimentally alter the activity of neurones *in vivo*. One is physiological manipulation where one can disrupt the physiological inputs to perturb synaptic input of a neurone. An example of this would be monocular depravation by lid suture (Hengen et al. (2013)). Another is pharmacological where a pharmacological substance can be applied to the brain to block the activity of a region (Echegoyen et al. (2007)). Last would be to genetically modify the intrinsic electrical properties of neurones to alter their excitability (Dey et al. (2013), Burrone et al. (2002)).

To accurately assess the molecular changes involved in plasticity *in vivo* I chose to genetically manipulate neurones. This approach has major advantages compared to the other approaches. In the physiological and pharmacological manipulations there is no way of distinguishing which cells were directly affected by the decreased activity and have adapted directly to the change. Also neuronal subtype specific manipulation is impossible. This is a problem as different cell types have different genetic profiles and will potentially use different strategies to adapt to the same changes. In contrast genetic manipulations can be directed to a specific type of neurone in a specific region of the brain. Also the affected neurones can be fluorescently labelled which allows isolation of affected neurones for genetic profiling experiments.

3.1.1 Genetically altering neuronal function in vivo

Genetic manipulation is a process where one introduces foreign genes to manipulate genetic expression in an organism. This was first demonstrated in mice by Jaenisch and Mintz in 1974 (Jaenisch and Mintz (1974)) where they injected simian virus 40 viral DNA into mouse blastocytes and detected the gene in adult mice. Gordon et al. (1980) developed this further and in 1980 created the first transgenic mouse line where the manipulation is passed down the generations (Gordon et al. (1980)). Since
then many transgenic mouse lines have been developed and were widely used as disease models (Gama Sosa et al. (2010)). However, transgenic mouse models have many complications. First, some of the genes which are of interest can lead to embryonic lethal phenotypes. Also the gene is altered globally which means that the resulting phenotypes in a region of interest can be due to the primary effect of modifications in the region or to secondary effects from the modifications in other regions. Lastly, there are complications with developmental effects of the gene.

Issues with transgenic mice which are described above can be overcome by the use of virus mediated genetic manipulation. Normal infectious virus works by recognising a host cell which is susceptible for infection. It then enters the cell and makes use of host cell’s machinery to replicates its own DNA. It then re-assembles itself in the cell and leaves the cell to infect new cells. The viral DNA can be manipulated to minimise the host immune responses and to deliver genes which can be expressed in the cell. Recombinant viral vectors and it can be derived from many different types of virus each giving different advantages and disadvantages (reviewed in Vannucci et al. (2013)).

In this study, I have used two viral vectors; lentivirus and adenosine associated virus (AAV). Lentivirus are a family of retroviridae which uses reverse transcriptase to stably incorporate its viral genome into the host genome. Most lentiviral vectors were developed from Human immune deficiency virus-1 (HIV-1), have reasonably large cloning capacity (up to 10kb pairs) and can infect non-dividing cells (Mátrai et al. (2010),Vannucci et al. (2013)). AAVs belong to family of the paroviridae and requires a helper virus, such as adenovirus, for replication. AAV is associated with low host immune response and can infect non- dividing cells (Giacca and Zacchigna (2012),Vannucci et al. (2013)). The transgene of interest can be cloned into the two types of viral vector and packaged into the viral coat by co-transfection with helper plasmids which codes for viral coats (see White et al. (2011) for lentivirus production, McClure et al. (2011) for AAV production). Viruses which are produced can be directly injected into the region of interest at the time of interest. With this strategy the genetic manipulation can be restricted to a region and can be delivered in adulthood, by passing the development issues of gene manipulation. Due to these reasons I chose to use virus mediated gene delivery method to alter the gene expression in vivo in adult neurones.
3.1.2 Activity dependent cell death

There are potential complications with decreasing neuronal activity. There is mounting evidence that neuronal activity has an impact on neuronal health. Traditionally people perceived neuronal health as a binary state, either alive or dead. However it has came to attention that the state of neuronal health is more complicated than the binary classification (Isacson (1993)). It has been demonstrated from a number of studies (reviewed in Isacson (1993)) that the state of neuronal neuronal health exists in a spectrum which is influenced by many factors. One of the factors which has been suggested to have influence on neuronal health is neuronal activity (Mennerick and Zorumski (2000), Corredor and Goldberg (2009), Bell and Hardingham (2011)). Neuronal activity can promote neuronal health by activating \( \text{Ca}^{2+} \) dependent neuro-protective pathways and suppressing apoptotic pathways. Indeed when the activity is blocked the neuronal health decreases as the neuro-protective pathways are not activated (Reviewed in Bell and Hardingham (2011)). This activity dependent neuro-protection mechanism involves genetic changes (Zhang et al. (2009)). Many experiments which demonstrated activity dependent cell death has been carried out in \textit{in vitro} culture system. Most of the \textit{in vivo} work has been done on developing brains (Xu et al. (2000), Papadia et al. (2008), Ikonomidou (1999), Galli-Resta et al. (1993)).

3.1.3 Experimental aim

To decrease the activity of neurones I have over-expressed Kir2.1. Kir2.1 is a subtype of inward rectifying potassium channel family (Nichols and Lopatin (1997)). It has been shown in many different neuronal types that over-expression causes decrease in neuronal activity (Karayannis et al. (2012), Burrone et al. (2002)). I have used viral vectors that express Kir2.1 with a mCherry C-terminal tag (Kir2.1-mCherry). Detailed procedure for the synthesis of the Kir2.1-mCherry transgene is given in White et al. (2011). Kir2.1-mCherry causes red fluorescent protein to express with the Kir2.1 channel thereby allowing assessment of the infection pattern \textit{in vivo}. I aimed to assess the infectious pattern for both lentivirus and AAV and determine which virus will be best for the down stream experiments. I did this by looking at mCherry expression 2 weeks after surgery which is the time point where electrophysiological and genetic study will be carried out.

As there are evidences that the decrease in neuronal activity causes adverse effects on neuronal health, I also wanted to assess the viability of the Kir2.1 over expressing neurones. This is important for later genetic profiling experiment as neurones under-
going apoptosis will have genetic changes related to the apoptotic process.

Therefore I aimed to characterise the infectious pattern of the two viruses to assess the rate of infection and neuronal health after infection to determine the most suitable virus to use for downstream physiological and genetic experiments.

3.2 METHODS

3.2.1 Experimental design

To investigate the possibility of cell death after Kir2.1mCherry expression I used a histological approach. 500nl of the virus was injected into the DG or CA1 with unilateral stereotaxic surgery. The precise dosage of lenti-virus injected will be given in the results sections where appropriate. The precise dosage of AAV were not determined but the same dosage was used all the way throughout the histological study and the following physiological and genetic study. The injection site was varied so that there is a similar number of injections in the right hemisphere and the left hemisphere. Mice were perfused 2 weeks after surgery and their brains were fixed and cryo-protected. 40μm Coronal free floating sections were prepared on a freezing microtome and stained with antibodies against neuronal cell bodies (NeuN 1:500) and mCherry (DsRed 1:400) to enhance the mCherry signal.

To assess the cell viability I first investigated the cell body layer after injection. The area of the studied region was calculated by measuring the area of NeuN stained cell bodies. I hypothesised that if cells undergo apoptosis there will be a loss of cell bodies in the virus injected region. Although this is a crude measurement of apoptosis only able to detect a large effect, this was a quick way of investigating apoptosis. The area of the virus expressing region was compared with the corresponding uninjected region in the opposite hemisphere. Also sectioning was performed so that the brain was completely level with the blade. This ensures that the measured area from the both hemispheres are comparable.

To further assess the cell viability with a higher sensitivity, Flouro jade C (FJC) and hematoxylin and eosin staining (H and E staining) were used to selectively label dying and dead cells. For all experiments, free-floating sections were prepared. Every 4th section was examined with immunohistochemistry.
3.2.2 Imaging, analysis and statistics

The sections were imaged on Nikon A1R FILM confocal microscope, Zeiss LSM510 confocal microscope and Leica TCS-NT. For area measurement, I used the measure tool in Image J. Length was also measured with Image J measure tool. All images were imaged at 10X objective lens for area analysis studies. Image J was also used for mean fluorescence measurement. The precise injection site was slightly different for all injections due to human error. As only the regions where the virus infection was detected was used for the analysis this results in a variability which is dependent on the variation in the surgery. To account for this variability corresponding area was measured from the noninjected side of the same mouse and the data was paired and paired statistics were used to calculate the statistical significance. Statistical test were performed using R (https://www.r-project.org).

3.3 RESULTS

3.3.1 Kir2.1-mCherry lentivirus infection may cause cell death in DG granule cell layer

I first wanted to evaluate the expression of Kir2.1-mCherry following in the granule cell layer of the DG after lentivirus injection. To determine the expression efficiency and pattern, Kir2.1-mCherry lentivirus was unilaterally injected in the the granule cell layer of the DG and the mCherry expression was determined by immunohistochemistry.

Example images from the Kir2.1-mCherry lentivirus injection are shown in figure 3.1. A punctate pattern of mCherry expression was observed in the membrane of DG granule cells. The expression was observed mainly in the soma but also in the dendritic regions. Also there were a distinctive proportion of cells which are expressing mCherry which indicates that lentivirus mediated Kir2.1-mCherry expression gives histologically detectable mCherry expression. The expression of mCherry was restricted to the injected side and there were no mCherry expression detected in the non injected side (Figure 3.1). These results show that Kir2.1-mCherry is expressed in DG granule cells following lentivirus injection. Typically around 30% of the cells were infected where maximum infection was seen and lower percentage of cells were infected in more dorsal and ventral area due to infection from spreading of the virus. The spread of the virus typically expanded 480µm in the coronal plane.
Figure 3.1: Lentivirus mediated Kir2.1-mCherry expression pattern in DG granule cells. Kir2.1-mCherry was expressed in DG granule cells by lentiviral injection. (A) Example images of injected side and corresponding non-injected side. DsRed antibody was used to enhance the mCherry fluorescence. The scale bar is 500µm. Region marked with white box is shown in (B) in higher magnification. Scale bar 100µm. Since the mCherry is fused to the Kir2.1 channel, mCherry is expressed in dotted pattern.

I next asked if Kir2.1 over expression by lentivirus infection causes cell death in the DG. I reasoned that cell death would lead to the loss of cell bodies in the DG granule cell layer. Therefore the area of the DG where the virus was injected was compared with the relevant area in the opposite hemisphere where no virus was injected. The spread of the virus did not cover the whole hippocampus and was variable from surgery to surgery. This may be due to the slight variation in the precise injection site. As the region where the virus did not spread are unlikely to be affected, the sections which did not express any mCherry was excluded from them analysis. The area across the granule cell layer, given by NeuN staining, was measured and the corresponding non injected area of the DG were measured from the opposite hemisphere. Measurement values from the images were averaged for each mice for both injected and non injected side (figure 3.2). A paired t test revealed that there is no sig-
Figure 3.2: *Analysis of area of DG after Kir2.1-mCherry lentivirus injection*. Average area of the DG injected with Kir2.1mCherry lentivirus the non-injected side was measured. Only sections that show virus infection were included in the analysis. Area of the upper blade and lower blade of the DG were analysed separately as well as the whole area of the DG. Area measurement from each mice are plotted in light purple and average over mice are plotted in dark purple. Paired t tests show that there is no significant difference in the upper blade, lower blade and combined blades of the DG (P=0.0637, P=0.4166 and P=0.2127). N=7 was used for this analysis.

A significant decrease in the area of the DG after the lentivirus injection (P=0.2127, N=7). The coordinates used for stereotaxic surgery was targeted to the upper blade of the DG. To further analyse the cell death in DG the top blade and the bottom blade were analysed separately. Neither blade differed significantly between infected and control sides (P=0.0637, P=0.4166, N=7). This indicates that Kir2.1 over expression does not cause enough cell death to be detected by the area measurement. However there is a trend towards reduction in the area of the upper blade in the injected side and with the p value nearing significance.

Cell death may be caused only in the neurones where the decrease in the activity is sufficient to trigger cell death. The degree of decrease in activity is dependent of how much Kir2.1-mCherry the neurone expresses in response to virus infection. This suggests that if the cell over expressed Kir2.1 at a larger magnitude this may be sufficient for causing cell death where as a slight over expression resulting in slight decrease in activity may not cause cell death. To test this hypothesis, higher titre stock of Kir2.1-mCherry lentivirus was prepared by scaling up the virus production reaction and by increasing the concentration rate. For a injection of 500nl, 6.5 X 10^3 transduction units were delivered for the high titred group and 1.2 X 10^3 transduction units for the low
titred group (results shown in figure 3.1 and figure 3.2). First, I wanted to confirm the difference in the degree of Kir2.1mCherry expression in the two different titres of the virus. Examples of degree of virus infection seen in two titres are shown in figure 3.3. Two different titers of virus show dramatic difference in the expression pattern under the same imaging conditions. The DG injected with high titre virus showed more mCherry fluorescence than the brain injected with low titre virus (results shown in figure 3.1).

With the different infectious pattern established in the two different virus stocks, I asked if there is a detectable amount of cell death in the hight titre virus injected DG. High titre virus was injected in the DG and the brain was harvested after 2 weeks. Sections were prepared and stained as before and the area of the DG was analysed in the same way. Results from the high titre virus injected group also show that there are no significant changes in the area of the DG after injection (figure 3.4) in all upper, lower and combined blades (P=0.0908, P=0.1393, P=0.0635, N=5). These results suggest that there are no cell death even after extreme over expression of Kir2.1-mcherry.
Figure 3.4: Analysis of area of DG after high titer Kir2.1-mCherry lentivirus injection. Average area of the DG injected with high titre virus and the non-injected side were measured. All analysis was done in the same was as the low titer virus injected group (results shown in figure 3.2). Area measurement from each mice are plotted in light red and average over mice are plotted in dark red. Paired t tests show that there is no significant difference in the upper blade, lower blade and combined of the DG (P=0.0908, P=0.1393, P=0.0635). N=5 was used for this analysis.

However, there were some individual samples where there was a sharp decrease in the area after injection. An example of this is shown in figure 3.5. Such examples were only observed in the high titre virus injected group.

Although there were no detectable cell death after Kir2.1-mCherry, some individual samples show a sharp decrease in the area. This raises concerns on the down stream experiments due to the reasons stated in the introduction section. In the lentivirus injection, the virus does not spread evenly throughout the DG. Instead, the virus is restricted to the point of injection so there are sections where there are high degrees of infection and there are sections where there are only a few cells infected by the virus. This means that within an experimental sample, there are DG sections which have high levels of infection and there will be sections from the distal regions from the injection site which have low levels of infection. As the criteria previously used included all the sections which showed any amount of infection, it may not be sensitive enough to detect small amounts of cell death which may be occurring, possibly in proportion to the degree of infection.

To test this idea I took all the individual images from all 12 mice (7 low titre virus injected and 5 high titre injected) and grouped them into 4 different groups ac-
Figure 3.5: Kir2.1-mCherry lentiviral injection can inconsistently cause cell death. There were some individual samples (N=2/5) where loss of granule cell layer was prominent. NeuN stain shows decrease of upper layer of DG granule cell layer and DsRed shows high expression of Kir2.1 in surviving cells. The scale bar is 500µm. Image in courtesy of Dr. Mel White.

cording to the degree of infection based on mCherry expression. 4 groups consisted of no-infection group, showing no infection in the whole blade, low infection with <10% cells infected, mid infection with <30% and high infection with anything above. The difference in area between the injected and non-injected side was calculated by subtracting the area of the non-injected side from the area of the injected side in the same section. All the data points were plotted according to the category of infection. The plot shows no obvious correlation between the degree of infection and the degree of cell loss (Figure 3.6).
Figure 3.6: Cell death caused by Kir2.1-mCherry lentivirus injection is not dependent on the degree of infection. Different degrees of infection in each section across all animals were categorised into 4 categories, no infection group, low infection group, mid infection group and high infection group. Examples of the infection pattern of 4 groups are shown in A. B) The change in area was determined by subtracting the area of the corresponding non-infected side from the area of the infected side. Average value of each category is shown in red. The plot shows no trend.

Together these results suggest that Kir2.1-mCherry lentivirus injection does not cause significant amounts of cell death even at high titres. However there is a trend towards reduction in the area of the DG in the injected area with some examples where there was a dramatic loss of cell body layer in the high titre virus injected group which remains a concern for downstream experiments. Also the measurement of cell death in this section was a very crude measure of cell death and this does not completely rule out the possibility of cell death occurring in small amounts.

I next wanted to confirm if the lentivirus infection or mCherry over expression has an adverse effect in the cell. This will test if the suggested cell death seen in Kir2.1 over expressing cells are due to lentivirus infection or due to the over expression of Kir2.1 and resulting effect of the over expression, suppression of activity. To test this, I made a control virus that only expresses mCherry. The mCherry lentivirus was injected unilaterally and the brain was sectioned as before. DG granule cells were stained with NeuN antibody and the mCherry signal was enhanced with DsRed.
staining. The titre of the virus used for this experiment was $2.1 \times 10^7$ TU/ml which is higher than the high titre virus used in the Kir2.1-mCherry injections.

![Image of staining results]

Figure 3.7: *Lentivirus mediated expression of mCherry in DG*. mCherry was expressed in DG granule cell layer by viral injection. A. Example images of injected side and corresponding non-injected side. NeuN stain was used to mark granule cell layer of the DG and DsRed stain was used to mark mCherry expression. The scale bar is 500μm. Region marked with white box is shown in B at higher magnification. The scale bar is 100μm

The virus injected brain was harvested as before and free floating sections was prepared. Example pictures of mCherry lentivirus injected DG are shown in Figure 3.7. The images show clear expression of mCherry on the injected side of the brain with no expression on the non-injected side. The mCherry also filled the soma and the dendrites uniformly unlike the mCherry expression pattern from Kir2.1-mCherry lentivirus injection. This is because the mCherry in Kir2.1-mCherry is tagged to the c-terminus of the Kir2.1 channel where as in the mCherry group it is freely dissolved. mCherry expression was restricted to the injected side of the brain. The native fluorescence of the mCherry in mCherry control virus was visible in the microscope unlike the mCherry signal from the Kir2.1mCherry virus. The sections were then analysed...
Figure 3.8: Lentivirus mediated expression of mCherry in DG does not cause cell death. Average area of the injected side and non-injected side was measured for each animal. Only sections that had virus infection were included in this analysis. Area of the upper blade and lower blade of the DG were analysed separately as well as the whole area of the DG. Paired t test shows that there are no significant changes in neither upper, lower nor combined (N=5, P=0.4518, P=0.2389, P=0.3225).

in the same way as the Kir2.1-mCherry injected brains. The results are shown in figure 3.8. There was no significant difference between the area of the injected and non-injected side of the DG. There were no differences in either the upper blade or the lower blade (P=0.4518, P=0.2389, P=0.3225, N=5). Moreover there were no examples where there were any signs of cell death occurring. This shows that the lentivirus infection itself nor mCherry protein does not cause cell death.

These results from Kir2.1-mCherry injection and mCherry injection demonstrate that there are some suggestions of cell death in DG granule cells associated with Kir2.1-mCherry expression. This cell death does not appear to be lentivirus dependent as mCherry virus does not cause cell death even though the injected dose of the virus was higher.

3.3.2 Kir2.1-mCherry lentivirus infection causes cell death in CA1 pyramidal cell layer

I next asked if the Kir2.1-mCherry lentivirus would cause cell death in other cell types. As I was interested in the homeostatic plasticity in CA1 pyramidal cells I investigated possible cell death related effects of Kir2.1-mCherry expression in the CA1 pyramidal cell layer. Kir2.1-mCherry was virally expressed in CA1 pyramidal cells.
500nl of low titre Kir-mCherry lentivirus (1.2 X 10^2 transduction units per injection) was injected into CA1 unilaterally. To control for any possible effects of lentivirus and mCherry expression on CA1 pyramidal cells, 500nl of the control mCherry virus (1 X 10^4 transduction unit per injection) was injected in to the CA1.

![Image of Kir2.1-mCherry injection and mCherry injection](image)

**Figure 3.9:** Lentivirus mediated expression of Kir2.1-mCherry and mCherry in CA1 pyramidal cells. Kir2.1-mCherry or mCherry was expressed CA1 by viral injection. Neurons were marked with NeuN stain and mCherry was marked with DsRed stain. A Comparison between the Kir21.-mCherry injected and corresponding non-injected CA1. B Comparison between mCherry injected and corresponding non-injected CA1. There is a slight disruption of the pyramidal cell layer in Kir2.1-mCherry injected CA1. This was not seen in the mCherry injected brain. The scale bar is 500µm and the scale bar in the insert is 50µm.

Example images from the CA1 injections are shown in figure 3.9. The pattern of mCherry expression in the Kir2.1-mCherry injected group and the mCherry injected group was similar to the pattern that was observed in the DG injected group. Kir2.1-mCherry injected CA1 shows punctuated expression of mCherry largely in the soma but mCherry injected CA1 shows uniform expression of mCherry in whole of the neurone. A small loss of pyramidal cell bodies was observed in the Kir2.1-mCherry injected CA1. This was not observed in all the mCherry injected sections which I looked at in this study. This suggests that the disruption in the CA1 is due to Kir2.1-mCherry expression and not due to the physical disruption of the CA1 by the injec-
tion pipette during the surgery nor by the lentivirus infection.

Figure 3.10: Analysis strategy allows selective measurement of infected area of CA1. In order to minimise the inclusion of non-infected area of the injected CA1 in the analysis, above procedure was taken for the analysis of all CA1 virus injections. NeuN and DsRed antibody was used to visualise pyramidal cell layer and mCherry respectively. The length of the infected region was measured and the area over the length was measured. The same length was used to measure the equivalent area of the non infected CA1. The individual area was divided by the length measurement to calculate the average thickness of the pyramidal cell layer. This was then averaged for each mice.

The area of the CA1 was quantified. Both Kir2.1-mCherry virus and mCherry virus did not spread through the whole of CA1 region. As there was no clearly discernible boarder between CA1 and CA2 I concentrated the analysis to the region of virus infection. First the spread of mCherry signal along the pyramidal cell layer was measured. The equivalent area was measured in the non injected hemisphere by taking the same length of CA1 and measuring the area. As the virus spread was variable between injection to injection, the area measured were divided with the length of the CA1 to calculate the average thickness of the CA1 pyramidal cell layer. Details of the analysis are shown in figure 3.10. By using this analysis method I was able to exclude the non-infected region of CA1 in the injected side, which would otherwise obscure
the changes in the infected region.

Figure 3.11: Kir2.1-mCherry expression via lentivirus injection causes cell death in pyramidal cell layer in CA1. The virally injected area of CA1 was measured. The equivalent area of non-injected CA1 was measured according to the length of CA1 that had virus infection in the injected side. Paired t test shows that there is a significant decrease in Kir2.1-mCherry injected CA1 (N=6, P=0.0202) but no significant decrease in mCherry injected CA1 (N=6, P=0.7296).

The average area of the injected and non-injected side was measured and average thickness of CA1 for each animal and plotted in figure 3.11. There was a significant decrease in the area of the injected side when compared to the non-injected side in the Kir2.1-mCherry injected mice (P=0.0202, N=6). However there was no significant difference found in the mCherry injected brains (P=0.7296, N=6). This shows that the CA1 pyramidal cells are susceptible to Kir2.1-mCherry induced cell death and this is not due to the lentivirus injection itself but due to the effect of Kir2.1 channel expression.

The results here indicate that Kir2.1-mCherry expression causes cell death in CA1 pyramidal cell layer. Indications of cell death were also observed in the DG in some examples but overall this was not statistically significant. This may be due to the cell type specificity of the Kir2.1-mCherry induced cell death but a more likely explanation is the difference in the analysis method approach taken for each region. The
method used for analysis of CA1 excludes the non infected region more efficiently than the method used for analysis of the DG where the whole blade was analysed even if the expression was restricted to parts of the blade. The CA1 analysis method was not suitable for DG analysis as the infection was usually spread out evenly across the whole area of DG.

3.3.3 Markers for cell death are not detectable following Kir2.1-mCherry lentivirus infection

Previous results have aimed to measure the amount of cell death by quantifying the absence of cell bodies after cell death. Although this would be able to detect large amounts of cell death which results in the change in the gross morphology of the brain region, it would not be suitable for detecting small amounts of cell death which may be scattered throughout the region of interest. To overcome this problem I investigated to see if markers of cell death may be visible in the hippocampus after Kir2.1-mCherry expression.

I tested two positive cell death marker candidates; H and E (Hematoxylin and eosin) staining and FJC (Fluoro Jade C) staining. H and E staining reveals structural changes more clearly after cell death (example of this use is seen in Martel et al. (2012)) and FJC staining is a commercially available marker of cell death which will label dying and dead neurones in green regardless of the apoptotic mechanism (Chidlow et al. (2009)). To check the sensitivity of the chosen markers I have used tissue from mice where the DG was lesioned with ibotenic acid injection. The injection was performed by Dr. Helen Ramsden.

Ibotanic acid induced lesion tissues shows clear loss of NeuN labeled granule cells in the granule cell layer of the DG. H and E stain shows morphological changes, shrunken nucleus with eosinophilic cytoplasm, when compared to non-lesioned tissue which is shown in figure 3.12 D. FJC stain shows clear labelling of dying cells in green (figure 3.12). From these results I concluded that the positive cell death markers chosen are reliable in detecting cell death.

I then tested the positive cell death markers on Kir2.1-mCherry injected DG. Images for injected and non-injected sides of the brain were taken for all time points and are shown in figure 3.12. There was no detectable signal for cell death with either method.
Figure 3.12: *H and E staining and FJC staining does not show cell death in Kir2.1-mCherry injected DG.* NeuN was used to mark granule cells. H and E stain was used to visualise the gross structure of the DG. The region denoted by white box is shown at higher magnification. FJC stain was used to stain the apoptotic cells. H and E stain and FJC were all able to mark ibotanic acid induced cell death in the DG. Kir2.1-mCherry was expressed by unilateral viral injection in DG. The results show that there are no positive markers of cell death present in the DG. Scale bar is 50µm.

I then tested the both positive cell death markers on lentivirus injected CA1. As seen previously, there was a disruption in the pyramidal cell layer of the CA1. However, there were very little or no cell death marker observed (figure 3.13). I identified 3 cells which show characteristics of apoptotic cell in H and E staining but there was no positive FJC staining.

These results show that there are limited evidences for cell death associated with lentivirus mediated Kir2.1-mCherry expression. There is a trend towards a reduction in the cell body area after Kir2.1 over expression in the DG and there is a significant
Figure 3.13: H and E staining and Flouro Jade C staining does not show cell death in Kir2.1-mCherry injected CA1. Kir2.1-mCherry was expressed by unilateral viral injection in CA1. NeuN was used to mark granule cells and DsRed was used stain for virus infection. H and E stain was used to visualise the gross structure of the DG and FJC was used to specifically target dead/dying cells. The results show that there are no cell death in Kir2.1-mCherry injected DG. However there was a disruption of the pyramidal cell layer in CA1.

loss of cell bodies in the CA1 pyramidal cell layer but there were little evidence of positive cell markers.
3.3.4  *Kir2.1-mCherry expression with AAV does not cause cell death*

Data so far shows mixed results. There appears to be loss of cell bodies associated with lentivirus mediated Kir2.1-mCherry expression especially in CA1 which are not due to lentivirus infection but Kir2.1-mCherry over expression. DG injection sometimes shows a decrease in the area of the granule cell layer after Kir2.1-mCherry expression but it was not statistically significant. However there were no positive cell death marker detected in neither CA1 nor DG. All positive marker used for this study has been shown to reliably detect cell death in ibotenic acid induced lesion tissue. Nevertheless, the possibility of significant cell death remains a concern for the experiments that follow. Therefore I investigated whether AAV might overcome these issues. I created an AAV which expresses Kir2.1-mCherry and mCherry. Due to the differences in the purification method for AAV and lentivirus, I hypothesised that the titres of AAV would be much higher (see materials and method chapter for detailed procedures).

The results show that, compared with the lentivirus injection, there are more infected cells in the DG of the brains infected with AAV Kir2.1-mCherry. All the sections analysed typically showed > 30% infection rate which was only observed at the region of highest infection in the lentivirus injected DG. The dotted expression pattern of mCherry was similar to that observed with lentivirus vectors injected DG. Axonal projections of the granule cells are observed in the contralateral non-injected hemisphere where as this was not visible in the lentivirus mediated expression of mCherry (Figure 3.14 and 3.15). The area of the DG was measured as described before. The results show that there were no significant change in the area of DG in Kir2.1-mCherry expressed DG and mCherry expressed DG indicating that Kir2.1-mCherry expression nor AAV infection does not cause cell death in DG. There were also no examples with obvious granule cell loss in all the examples that I examined (N=4 brains, 5 sections per brain, Figure 3.16).

I next asked if Kir2.1-mCherry causes cell death in CA1 pyramidal cells. Again, Kir2.1-mCherry and mCherry expressing AAV was injected unilaterally into CA1 of the mouse and histologically analysed after 2 weeks. The brain was perfused 2 weeks after surgery and the sections were prepared as before. The images show no apparent signs of cell death in the pyramidal cell layer of the CA1 in response to Kir2.1-mCherry expression (figure 3.18). The analysis of the area after injection was carried out as before (figure 3.10). The analysis shows that there are no significant change in the area of CA1 after Kir2.1mCherry or mCherry expression by AAV (P=0.427,
3.4 Discussion

The aim of experiments presented in this chapter was to determine if Kir2.1-mCherry over expression causes cell death through suppression of neuronal activity. The experimental design for the thesis was to induce neuronal plasticity in response to
Figure 3.15: High magnification of mCherry expression pattern in AAV Kir2.1-mCherry
Kir2.1-mCherry and mCherry was expressed by AAV injection in DG. High magnification image from Kir2.1-mCherry and mCherry AAV injected DG are shown. Arrow shows two granule cells one infected with the AAV and one un infected. Axonal projection is more clearly seen. Scale bar is 50µm.

Figure 3.16: Kir2.1-mCherry and mCherry expression via AAV infection in DG does not cause cell death Area of virus injected DG was measured and compared to corresponding non injected DG area. All sections from one mouse was averaged and shown in light red. Average of all mice are shown in red. Paired t test shows that there are no significant differences between the area of injected DG and non-injected DG for both Kir2.1-mCherry and mCherry (N=4 P=0.5206, N=3 P=0.4030)
suppression of neuronal activity and to analyse genetic changes. If the neurones are undergoing apoptosis in response to the suppression of activity this would mean that the genetic changes will be associated with the apoptotic process and not plasticity. As there were evidences that decrease in activity may cause vulnerability in neurones (Bell and Hardingham (2011)), I wanted to test if Kir2.1-mCherry over expression causes cell death in DG granule cell and CA1 pyramidal cells. This will ensure that the genetic changes detected will be due to the homeostatic changes and not due to the cell death process. One other reason to characterise the anatomy after infection is to determine the efficiency of the infection. Anatomical analysis would characterise patterns of expression and ensure that the virus can induce genetic changes in vivo.

I have tested two different viruses; lentivirus and AAV. Lentiviral vectors have the advantage that they can express larger transgenes whereas AAV causes less immune reactions in vivo (Vannucci et al. (2013)). Lentivirus Kir2.1-mCherry shows that there
are detectable levels of cell loss in the CA1 pyramidal cell layer but not in DG granule cell layer. The analysis of area of the cell body layer is a very crude measurement of cell death so there are high possibilities that the more subtle cell deaths will be missed by using this technique. However, neither H and E staining and FJC staining detected cell death. As these two methods have previously been used as cell death markers (Martel et al. (2012) and Chidlow et al. (2009)) these results show the absence of cell death more clearly. In the lentivirus injected DG there were some examples where very prominent cell loss was observed in high titre virus injections. However this was not observed in all the injections I have performed in this study with the low titre virus stocks. Further analysis revealed no relationship between the level of virus infection and the degree of cell loss. The reason why CA1 has a significant decrease in area after Kir2.1-mCherry expression and not DG may be due to the pattern of infection and analysis methods. Infection pattern is to be more concentrated in around a small region in CA1 then in DG where the infection is seen more sparsely. More-
over, the analysis method used in analysing the area of CA1 after injection allows focused analysis in the virus infected region of the CA1 where as in DG, due to the sparse infection, this was not possible. Experiments with the control virus (lentivirus expressing mCherry alone) did not show any signs of cell loss suggesting that the cell loss is due to Kir2.1 expression rather than high titre infection of lentivirus. However there were no signs of positive cell death markers in the Kir2.1-mCherry injected CA1 thus complicating the interpretation of the area measurement result.

Following injections of AAV Kir2.1-mCherry, there were no signs of cell loss in either CA1 or DG. There was a massive increase in the number of infected neurones in the AAV injected brain compared to the lentiviral injections. This qualitative observation was confirmed by flow cytometry experiments where AAV injected DG showed dramatically larger mCherry positive cell numbers than the lentivirus injected DG (from around 100 mCherry positive cells in lentivirus injection to around 3000 mCherry positive in AAV injections). This results will be presented in chapter 5 of this thesis.

Although there is mounting evidence that decrease in activity will lead to cell death, most of work has focused on the in vitro culture system and developing brain (Bell and Hardingham (2011), Corredor and Goldberg (2009)). Results from AAV suggest that in adult neurones in vivo decrease of activity does not cause cell death. However the results from lentivirus is interesting in that it sometimes does cause cell loss. One possible reason why this may be is that in a dense tissue the lentivirus does not spread as well as AAV due to its bigger particle size. As well as the bigger particle size, difference in the preparation method cause lentivirus to be more viscous than the AAV which may contribute to confinement of the virus around the injection site. This could result in more infection per cell in the lentivirus injected brain than the AAV injected brain as the virus is more confined. If the lentivirus infects fewer cells but each cell at a higher density, this may cause a bigger drop in resting membrane potential and the input resistance of the cell which the cell may not be able to adapt to. However it is worth noting that the electro-physiological characteristics are similar between the lentivirus and AAV infected cells (the results are presented in chapter 4 of this thesis). This however would not be a full proof as heavily infected cells in the lentivirus infected brain could have undergone apoptosis before the electrophysiological study and only the cells with less infection (similar levels with the AAV injection) could have survived.
3.4 Discussion

3.4.1 Conclusion

In conclusion, the lentivirus gives mixed results in terms of cell death profiles. Although the experimental evidences provided in this chapter suggests no cell death, there are evidences of cell death in the DG and CA1 with the lentivirus. Moreover the infected cell numbers are much lower in the lentivirus injected brain when compared to AAV injected brain. Moreover, AAV was not associated with cell death in all the samples I have tested. Therefore I concluded that the AAV is more suitable virus for downstream experiments due to higher infection rate and less adverse effect on the infected cells. I have used AAV for all down stream electrophysiological and genetic profiling experiments described in chapters 4 and 5.

3.4.2 Future work

The suggested cell death in the lentivirus group needs to be confirmed with more experiments and more markers. I have used H and E staining and FJC as a positive cell death marker but there are other widely used cell death markers such as tunnel staining. It would be informative to test other cell death markers. Also as the cell death were only observed in the high titre virus, even higher titre stock can be prepared to increase the effect for more reproducible cell death with Kir2.1/-mCherry lentivirus infection.

To confirm if the cell death seen in CA1 pyramidal cells in lentivirus injected brain are indeed activity dependent, activity dependent neuro-protective pathways can be genetically ‘turned on’ while Kir2.1-mCherry is expressing. One of the pathways is PI3K-Akt pathway which is strongly activated by NMDA activation (Hardingham (2006), Bell and Hardingham (2011)). Activation of PI3K-Akt pathway suppresses apoptosis in a number of ways. One is by phosphorylating Forkhead box transcription factor, class O (FOXOs) and suppressing the activation of down stream genes which are involved in apoptosis. Other mechanisms include activating survival genes via CREB or NF-κB and by directly inhibiting the apoptotic machinery (Brunet et al. (2001)). If the cell death in Kir2.1-mCherry is indeed activity dependent it would be prevented by activating the activity dependent neuro-protective pathway. Akt can be mutated by adding src myristolation signal which is 14 amino acids which results in constitutive activity (Takeuchi (1996)). There are evidences that Kir2.1-mCherry causes cell death in vitro which can be rescued by expressing myristolated Akt (Unpublished data in collaboration with Prof. Giles Hardingham). Virally co-expressing
Kir2.1-mCherry and myristolated Akt would show if the cell death is activity dependent. This would be a direct test of the cause of cell death and if it is indeed rescued and there are no disruption in the layer it will reinforce the evidence for cell death.
4 ELECTROPHYSIOLOGICAL ANALYSIS OF CELL PROPERTIES
AFTER KIR2.1 OVER EXPRESSION

4.1 INTRODUCTION

4.1.1 Studying mechanisms of neuronal homeostasis

It has been demonstrated in many different preparations that there exists homeostatic plasticity which is involved in stabilising neuronal network and prevents activity over run by other plasticity mechanisms such as hebbian synaptic plasticity (for review see Marder and Goaillard (2006), Turrigiano (2012), Davis and Müller (2015), Zhang and Linden (2003)). Much of the work has focused on cultured preps for technical ease during experiments (Turrigiano et al. (1998), Burrone et al. (2002), Sutton et al. (2007)). Although culture systems are valuable in determining precise physiological mechanisms and molecular mechanisms, culture system provides a very different environment for the neurones and so it is not clear the extent to which results are applicable to in vivo settings. Recently, there has been a focus in investigating the homeostatic plasticity ex vivo and in vivo (Desai et al. (2002), Echegoyen et al. (2007), Hengen et al. (2013), Keck et al. (2013)) but much work has relied on removing inputs, typically through eye suture, or infusing neuronal blockers in live animals. Although these approaches can serve as a good means for investigating general adaptation of a neuronal population, it is not clear which of the cell would be affected by the manipulation. As neuronal circuits consist of many types of cells with different functions global blockade of activity may mask some important adaptation mechanisms.

A way to overcome this issue is to target manipulations to defined populations of cells. Goold and Nicoll (2010) used sparse bolistic transfection technique to express channelrhodopsin 2, a light gated proton pump (Nagel et al. (2003)), in CA1 pyramidal cells in organotypic cultures. As the transfection efficiency was not 100%, the authors were able to activate a sub population of cells in CA1 and sample from an activated cell and a near-by unactivated cell as a control group. The authors then applied electrical stimulation with pharmacological agents to investigate the specific cellular changes after homeostatic plasticity. Burrone et al. (2002) and Hou and Zhang (2008) expressed Kir2.1 in hippocampal cultures to suppress neuronal activity in Kir2.1 ex-
pressing cells. Kir2.1 channel belongs to a family of inward rectifying potassium channels. The channels are constitutively active and have a role in maintaining the membrane potential of neurones in which they are expressed (Doupnik et al. (1995)). Over expression of these channels has been used to suppress neuronal activity in many neuronal subtypes and in different preparations (Johns et al. (1999), Burrone et al. (2002), Hou and Zhang (2008), Dong et al. (2006) and Yu et al. (2004)). Burrone et al. (2002) particularly studied the adaptation in the postsynaptic cell after suppression of neuronal activity during and after development by directly studying the Kir2.1 expressing cells. Hou and Zhang (2008) studied adaptations in individual postsynapses after suppression of activity by studying the synapses that are from the Kir2.1 expressing cells and comparing it to the synapses that are from untransfected cells. Both studies were carried out in cell cultures. The above studies demonstrates the advantages of targeting individual cells to manipulate activity within a neuronal circuit as opposed to the removal of inputs or application of TTX to completely suppress all activity.

I chose to suppress neuronal activity in vivo by over-expressing Kir2.1 in hippocampal excitatory neurones (DG granule cells and CA1 pyramidal cells) to study the post synaptic molecular mechanisms of neuronal adaptation. There is evidence of cell type differences in the mechanisms of adaptation with pharmacological blockade of neuronal activity in slice cultures but this has not been shown in vivo (Kim and Tsien (2008)). The detailed description of the tool development and characterisation of the tool is presented in the previous chapters and in White et al. (2011). Here I will present the electrophysiological evidences for the Kir2.1 over expression and adaptation. As work presented in this chapter is a direct continuation of the previous work that was done in Nolan lab (Unpublished) with DG granule cells, I will present in the introductory section the previous data obtained in the lab. All the experiment and analysis presented in this section were carried out by Dr. Paul Dodson, Dr. Cian O’Donnelle and Dr. Melanie White.

4.1.2 Over expression of Kir2.1 reduces excitability in DG granule cells

Inwardly rectifying potassium currents are involved in the forming of resting membrane potential and membrane conductance. We hypothesised that the over expression of Kir2.1 in neurones in vivo will change the membrane properties of the cell as seen in Johns et al. (1999) and Burrone et al. (2002). Kir2.1-mCherry was expressed with lentiviral infection in 4-6 weeks old male C57/BL6 mice. The mice were scarified 2 weeks after the stereotaxic surgery and 400µm acute coronal slice were prepared.
The lentivirus infected cells were identified with mCherry expression and the basic electrophysiological properties were measured in current clamp mode. As a control group, nearby uninfected cells were used.

The current clamp results show that the resting membrane potential of the Kir2.1 infected cells were significantly lower than the nearby uninfected cells. The input resistance of the infected cells are significantly lower than the neighbouring uninfected cells. This is also seen in the example traces where more current was needed to raise the membrane voltage by about 5 mV. The membrane time constant was also significantly lower than that of the uninfected cells (figure 4.1).

Kir2.1 over expression causes changes in the basic membrane properties of the cell in vivo. How do these changes in basic properties of the cell impact the input output properties of DG granule cells? In vitro experiment by Burrone et al. (2002) has shown that the expression of Kir2.1 causes depression of activity in old cultured neurons acutely after expression. This then leads on the homeostatic adaptation and normalisation of spiking rate. Experimental confirmation in vivo was challenging due to uncertainty in time point of gene expression after stereotaxic surgery. To overcome this, a computational model of the hippocampal network was used to estimate the excitability of the cell by using the cellular properties measured from the current clamp experiment.

The computational model used for this work is described in Ferrante et al. (2009). This model consists of a single granule cell receiving external inputs which represents entorhinal cortex inputs to the hippocampus. The projection strength are determined by synapse number and weight. The granule cell also receives feedforward inhibition from the molecular layer perforant pathway interneurones which is also controlled by the entorhinal cortical inputs (figure 4.2). These properties make the model biophysically and anatomically plausible. The model was adjusted according to the physiological properties measured in current clamp experiment.

The results from the simulation shows that given the changes in basic properties of the granule cells after Kir2.1 over expression, larger synaptic stimulation is needed for the Kir2.1 over-expressing cell to reach the threshold compared to the control granule cells (figure 4.3). For the simulations the MOPP interneuron and its feedforward inhibition was removed. This decrease in excitability is consistent with the acute suppression of activity seen after Kir2.1 over-expression in Burrone et al. (2002).
Figure 4.1: Analysis of sub-threshold characteristics of Kir2.1 over expressing DG granule cells. Sub-threshold properties of the infected and uninfected DG granule cells were measured with current clamp. (A - C) Example traces with positive current injection for Kir2.1 over expressing (A) and uninfected (B) cells are shown. (C) Two voltage traces were overlapped with respective current injection trace in the bottom panel. The initial rise phase of the voltage trace is also shown on the top panel. (D - F) Selected electrophysiological properties are measured. Vm (resting membrane potential) was significantly lowered by Kir2.1 over expression (-82.41 ± 0.63mV, -77.45 ± 0.79mV, P<0.0001 N= 65, 83. Kir2.1 and non infected respectively). Input resistance of the cell was significantly lower by Kir2.1 over expression (108.35 ± 9.10MΩ, 211.23 ± 7.18MΩ, P<0.0001, N=65, 83. Kir2.1 and non infected respectively). The time constant was also significantly lower by Kir2.1 over expression (5.77 ± 0.49ms, 14.1 ± 0.66ms, P<0.0001, N=65, 83 Kir2.1 and non infected respectively). All experiment are preformed by Dr. Paul Dodson

4.1.3 Adult DG granule cells in vivo adapt to depression of activity

The data from the model simulation suggest suppression of excitability in Kir2.1 overexpressing cells in absence of any adaptation in the system. Therefore, measuring the excitability of granule cells after 2 weeks of infection would give us information on whether the cells have adapted to the suppression of activity. We hypothesised that if there is adaptation, the excitability of the virus infected neurones would be similar
Figure 4.2: Schematic of the model used in Ferrante et al. (2009). The model consists of entorhinal cortical inputs which is determined by its number and the weight of the synapses. The model has two components, granule cells and molecular layer perforant pathway interneurons (MOPP) which receives inputs from the entorhinal cortex. There is also a feed forward inhibition pathway from MOPP cell to the granule cell. Figure from Ferrante et al. (2009) figure 1 panel A.

to the nearby uninfected neurones. However if there is no adaptation, the excitability of the virus infected neurones will be reduced compared with the nearby uninfected neurones as the model predicts.

To investigate excitability, the cells were electrically stimulated with silver coated stimulating electrode and the response was measured in current clamp mode. To maximise statistical power by enabling paired comparisons, a nearby non-infected cell was simultaneously patched with an infected cell. We reasoned that the nearby cells would be receiving very similar input to the infected cells thus reducing the difference caused by cells receiving different inputs from the stimulation. The cell was held in current clamp mode with no somatic current injection. The synaptic inputs were then stimulated with stimulating electrode in the third molecular layer and its voltage response was measures in the soma. Stimulation intensity at which the uninfected cell fires an action potential was denoted as threshold. The stimulus was then changed in small incremental or decremental steps until the infected cell fires an action potential. The difference of the two threshold for action potential firing was calculated and normalised to their sum. If there is no adaptation the Kir2.1 over-
Figure 4.3: Simulated excitability of Kir2.1 expressing granule cells. Changes in excitability in resulting from Kir2.1 expression was estimated by biophysically and anatomically plausible model of granule cell receiving inputs from entorhinal cortex. Properties of the granule cell was altered as measured in figure 4.1. Increasing the synaptic stimulus that the cell receives reveals that the excitability is estimated to be suppressed in neurones with Kir2.1 over expression. The simulation is done by Dr. Cian O’Donnello expressing cell would require more synaptic current for action potential firing due to the decrease in excitability pushing the difference/sum value to a positive value.

The results show that in contrast to the estimations from the model, the threshold for spike firing remains unchanged after Kir2.1-mCherry expression (figure 4.4 B). This suggest that there is homeostatic adaptation in vivo in adult DG granule cells after 2 weeks to adjust the excitability to the same level as the control cells.

4.1.4 Adaptation in DG granule cell does not involve synaptic changes

One of the ways that a neurone may adapt to the change in excitability is through synaptic scaling (Turrigiano et al. (1998), Turrigiano (2008)). It has also been shown that mature pyramidal cells in culture adapt to depression of activity induced by Kir2.1 over expression by increasing the frequency of the mEPSCs Burrone et al. (2002). In order to determine if the DG granule cells in vivo also use this mechanism, synaptic inputs to infected cells were investigated with voltage clamp and electrical
Figure 4.4: Adaptation of excitability in Kir2.1 expressing granule cells in DG. (A) Pair of infected and uninfected cells were simultaneously patched and electrically stimulated. (C) Example traces obtained from such pair are shown. Electrical synaptic stimulation results in voltage response and the threshold value for the pair was set by the intensity when the uninfected first fired an action potential. The stimulation was increased/decreased in small increment steps until the infected cell fires an action potential. (B) Analysis of excitability. The difference between the two threshold values were divided the sum of the two threshold values (threshold being 1). Red arrow indicates estimated value calculated by the predictions. The physiological threshold measured were similar between infected and uninfected cells. All experimental work was done by Dr. Paul Dodson.

stimulation. As before, a pair of infected and uninfected cells was patched simultaneously and was electrically stimulated to minimise experimental noise discussed previously. Picrotoxin was bath applied to prevent activation of GABA-A receptors from activation of inhibitory axons. The voltage of the patched cells were clamped at -70 mV to maximise AMPA current which has previously been suggested as a target for homeostatic changes (Turrigiano et al. (1998)). The internal solution also contains QX314, barium chloride and TEA. This blocks potassium currents and voltage gated
sodium channels allowing isolation of AMPA currents.

Figure 4.5: Homeostatic adaptation does not involve synaptic scaling. (A - B) Example trance from electrically evoked EPSC (A) and spontaneous EPSC (B). (C) The difference between the amplitude of the evoked EPSC from infected and uninfected cell was divided with the sum of the two amplitudes. The average of this value was 0 indicating that there were no difference (t test, P=0.905, N=10 pairs) (D) Two consecutive stimulus was given in a short period (50 ms). The ratio between the first and the second was taken to calculate the paired pulse ratio (PPR). PPR was analysed in the same way and there was no difference between the infected cell and non infected cell (t test p= 0.983, N=10 pairs) (E - F) The frequency of the spontaneous EPSC (sEPSC) and amplitude was taken and the difference over sum was calculated. Both show no change (t test, P=0.911, P= 0.654 frequency and amplitude respectively, N= 5 pairs).

All experimental work was done by Dr. Paul Dodson.

The results show that there are no synaptic changes in Kir2.1 over expressing neurones after 2 weeks. There were no difference in the evoked synaptic response compared to the nearby uninfected neurones. There was also no difference in the amplitude or the frequency of mEPSCs. Paired pulse ratio analysis of evoked EPSPs shows that the quantal release probability was not altered.

If the neurones do not adapt through synaptic scaling, what other strategy do they use? An alternative strategy for adaptation is modification of intrinsic excitability.
Figure 4.6: Change in current to voltage ratio in membrane voltage near threshold. (A - B) Example traces from voltage trace in response to artificial EPSC. The amplitude of artificial EPSP per current injection was plotted at the bottom panel. From the threshold value, aEPSC -10pA was delivered and in -10pA decremental steps down to threshold -40pA. The slope of voltage step calculated from this region was plotted in (C). Kir2.1 shows increased when compared to the infected population (P=0.0914, T test). All experiments were done my Dr. Paul Dodson.

(Desai et al. (1999), Nataraj et al. (2010)). To closely look at the intrinsic properties involved in synaptically evoked excitation, aEPSC (artificial EPSC) were delivered with current injection into the soma. The amplitude of the aEPSC needed to cause the cell to fire was determined by injecting incremental currents. Once the threshold was determined, the amount of current was altered in 10 pA decremental steps to 40 pA below the threshold. This allowed the analysis of input resistance at a membrane voltage near the spike threshold. The slope of voltage change during these current steps was calculated. Kir2.1 infected neurones has a trend towards increased slope in this region when compared to uninfected population (figure 4.6). This indicates that Kir2.1 expression may cause cells to adapt by changing its input output relationship to stimulus when the membrane voltage is near the threshold.

4.1.5 Experimental aim

The experiments summarised above show that Kir2.1 over expression causes changes in intrinsic properties in DG granule cells. These changes are predicted to cause de-
creased excitability by neuronal model of DG granule cell but this was not the case in slice physiology experiments hence suggesting adaptation. The mechanism of this adaptation did not involve changes in synaptic processes. Initial experiments suggests that the changes may involve input output relationship at a specific membrane potential. These initial experiments have used lentiviral vectors. In chapter 3 of this thesis I showed that this approach is potentially associated with cell death. It is also limited for analysis of gene expression as only a few cells were isolated limiting the amount of material available for gene profiling experiments. The results from chapter 3 also suggests that AAV has higher infection rate and also no detectable associations with cell death. Therefore I set out to establish whether expression of Kir2.1 from AAV infection could also be used to manipulate excitability of granule cells in the DG. I first wanted to confirm the electrophysiological changes seen after Kir2.1 overexpression with AAV and link it with the results from the lentivirus infected cells. To show this I decided to replicate the current clamp experiment with lentivirus (figure 4.1) but using AAVs.

I also wanted to investigate the cell type differences in vivo. Work from acute slices has shown that there are differences in the homeostatic adaptation in different synapses (Kim and Tsien (2008)). This was not demonstrated in vivo. To this end I have also expressed Kir2.1 with AAV infection in CA1 pyramidal cells and investigated possible adaptation. Previous result shows that application of TTX in adult CA1 pyramidal cell causes adaptation through increase in the frequency of mEPSCs (Echegoyen et al. (2007)). I aimed to confirm the changes in electrical properties after Kir2.1 over expression with current clamp and aimed to measure synaptic properties after AAV infection to show if there are any adaptation after 2 weeks in CA1 pyramidal cells.

In other experiments for this study (anatomical studies presented in chapter 3 and genetic study presented in chapter 5) mCherry control virus infected cells were used as a control group. However nearby uninfected cells can be advantageous in that they can be sampled from the same slice during the experiment reducing the batch effect. Also are in a very similar network environment receiving similar synaptic inputs which adds sensitivity to the stimulation experiments. Due to these reasons I have chosen nearby infected cells as a control group for this study.
4.2 METHODS

4.2.1 Current clamp protocols and analysis

To measure the basic electrophysiological properties of a cell, such as its resting membrane potential, input resistance, time constant and rheobase, I used current clamp recordings of membrane potential responses to injection of various currents into the cell. Bridge balance compensation was used and the maximal series resistance was 30 MΩ. The resistance of the recording electrode was 4 - 7 MΩ.

For determining the resting membrane of the cell I held the cell at 0 pA current while measuring the membrane voltage for 1 second. The membrane voltage readings obtained during this second was averaged to obtain the resting membrane potential of the cell. To calculate the input resistance and the time constant, the cell was held at various current from -80pA to 80pA in 20pA increment steps of 3 seconds each while the membrane voltage was measured. The cell was held at 0 pA for 2 seconds in between each current injection step.

The input resistance was determined by taking the steady-state voltage response and dividing it by the injected current. Input resistance was calculated from both the positive and negative current steps. The steady state voltage response did not change during the 4 second current injection period. Membrane time constant was calculated by fitting an exponential function to the membrane potential between 5ms and 100ms from the start of the current injection.

For calculating rheobase, positive current was injected from 20 pA in 20pA incremental steps for 4 seconds until the cell fires the first action potential. 2 seconds of rest period (when there were no current injected) was given in-between each current injection steps. The first current step at which the neurone fires an action potential was taken as the rheobase of the neurone.

All data was collected at 20kHz sampling rate. Low pass filtering of 10kHz were applied. All recordings were made at 35° C. All analysis was done on Igor Pro with custom written scripts (Nolan et al. (2004)) and the statistics were done on R (R-Project) or excel (Microsoft).
4.2.2 Voltage clamp protocols and analysis

To measure synaptic currents I used the voltage clamp recording configuration. To focus the study on excitation, I blocked GABAa current with extracellular picrotoxin (50µg/ml). To cancel out the difference in the current flow due to the expression of Kir2.1 channels I used Barium and TEA as intracellular potassium channel blocker. I also blocked voltage gated Na+ channel with QX314 to prevent the cell from firing an action potentials. For all voltage-clamp experiments cells were held at -70mV. Series resistance compensation was applied and the maximal series resistance was 30MΩ. A pair of virus infected cell and nearby uninfected cells were patched simultaneously. Data collected from the two cells were analysed with a paired design. To ensure that both cells receive similar inputs from the stimulating electrodes two cells were kept in 5 cell distance from each other.

To focus the study on the excitatory adaptations all the inhibition was blocked by picrotoxin. Also as blocking inhibition with picrotoxin causes CA3 induced seizure activity (Hablitz (1984)) so I removed the CA3 from coronal slices with micro-dissection. The schematic representation of the experiment and the image of the organisation of the setup during the experiment is shown in figure 4.7. I electrically stimulated CA1 pyramidal cells by stimulating schaffer collateral pathway of the hippocampal network. For this, stimulating electrode was placed in the middle of the stratum radiatum lateral to the region where the patched cells were located. The stimulating intensity and the position of the stimulation electrode was altered so that the synaptic current is clearly visible in the recorded cells. The internal solution also contained QX314, barium chloride and TEA to block voltage gated sodium channels and potassium channels which would mask the AMPA current.

In addition, biocytin was added to the internal solution used in the experiment and all neurones recored were reconstructed after the experiment. This was to reconfirm the infectious pattern of the recorded cells. Also the morphology of the recorded cell was used to control for recording non pyramidal cells. Only the pair of pyramidal cells with infected and uninfected cells were used for downstream analysis.

Electrical pulses of duration 0.5ms at an amplitude where clear evoked synaptic current of 100pA or more was given following a 400ms rest and the membrane potential was stepped to -120mV for 200 ms then back to -70mV. This negative holding potential was to reveal the transient current for quality check of the voltage clamp. The shape of the current was assessed for sharp transient currents and the compen-
Figure 4.7: Experimental setup for CA1 paired patching experiment. (A) Schematic representation of CA1 paired patching. A pair of virus infected and nearby non-infected were patched simultaneously while schaffer collaterals are stimulated by electrically stimulating electrode. The slice was kept under picrotoxin to block inhibitory inputs to the cell and CA3 was removed to prevent uncontrolled activation of schaffer collaterals. (B) image taken from an experiment showing all the components.

sation value was changed if necessary.

To measure spontaneous events cells were simply held at -70mV while continuously recording the current for 3 minutes. Paired pulse ratios were measured by stimulating the schaffer collateral at 20Hz both 0.5ms long.
All data was collected at 20.513kHz sampling rate. Low pass filtering of 5kHz were applied. All recordings were made at 35° C. All analysis was done with Igor Pro with Neuromatic software. Statistics analysis used R (R-Project) or excel (Microsoft). The spontaneous events were detected with custom written igor script and with custom defined threshold levels for each cell. All the detected events were manually confirmed to reduce false positive rate. The spontaneous events were detected with custom written igor script and with custom defined threshold levels for each cell. All the detected events were manually confirmed to reduce false positive rate.

4.3 RESULTS

4.3.1 Electrophysiological properties of AAV-Kir2.1-mCherry infected DG granule cells

As all the previous electrophysiological characterisation of Kir2.1 over expressing DG granule cells (described in the introduction of this chapter) was carried out on lentivirus infected cells I first confirmed whether the electrophysiological changes induced by AAV infection is comparable to the lentivirus mediated changes.

AAV Kir2.1-mCherry virus was injected into the DG and the brain was harvested 2 weeks after injection. Acute slice was prepared and virus infected cells and nearby uninfected cells were analysed. The results show that there is a decrease in the resting membrane potential, input resistance and membrane time constant (resting membrane potential = -80.68 ± 2.52 mV and -76.91 ± 1.41 mV, input resistance = 92.54 ± 13.12 Ω and 288.18 ± 28.68 Ω, time constant = 3.42 ± 0.44 (ms) and 10.37 ± 0.78 (ms). Kir2.1 expressing and nearby uninfected cell respectively). Statistical significance of the changes were tested by student’s t test and the result shows that the differences in the input resistance and the time constant are statistically significant (p value = 1.032e-05, 8.956e-06, Input resistance and time constant repectively). However, the difference in the resting membrane potential was not statistically significant (p value= 0.2097 figure 4.9). All were tested with non-paired t test.

The changes in membrane properties via to AAV mediated Kir2.1 over expression were compared with the equivalent data obtained from lentivirus mediated Kir2.1 over expressing cells (table 4.1). The resting membrane potential and the input resistance measured from two groups were not significantly different (P= 0.3457 and p= 0.4741 respectively). However, the membrane time constant was significantly different (P=0.0471). Non-paired t test was used to calculate the statistical significance.
Figure 4.8: AAV mediated Kir2.1 expression suppresses excitability of DG granule cells (A) Example of voltage traces from Kir2.1-mCherry expressing cells and nearby uninfected cells. Current injection that depolarised the cell by 10 mV is shown. 40pA was injected to the Kir2.1 expressing cell and 20pA was injected to the uninfected cell. Region in blue box is shown in far right panel. (B - D) Sub threshold electrophysiological characteristics of the DG granule cell after Kir2.1 expression. Resting membrane potential was lowered by Kir2.1 expression (-80.68 ± 2.52 mV and -76.91 ± 1.41 mV, p value 0.2097, N=13,12 Control,Kir). Also, input resistance and time constant was significantly lowered in response to Kir2.1 expression (92.54 ± 13.12 MΩ and 288.18 ± 28.68 MΩ p value = 1.032e-05, 3.42 ± 0.44 (ms) and 10.37 ± 0.78 (ms) p value = 8.956e-06, input resistance and time constant respectively, N=13,12 Control,Kir).

<table>
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<tr>
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<th>AAV (N=12)</th>
<th>Lentivirus (N=64)</th>
<th>P value</th>
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<tbody>
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<td>Resting membrane potential</td>
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<td>-82.41</td>
<td>0.3457</td>
</tr>
<tr>
<td>Input resistance</td>
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</tr>
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<td>Membrane time constant</td>
<td>3.42</td>
<td>5.77</td>
<td>0.0471</td>
</tr>
</tbody>
</table>

Table 4.1: Statistical comparison between electrophysiological properties of lentivirus infected cells and AAV infected cells. Comparison reveals that there are no significant differences between the resting membrane potential and input resistance of the lentivirus or AAV infected DG granule cells. Membrane time constant was significantly different with p value of 0.0471. Sample numbers used were 12 for AAV infected cells and 64 for lentivirus infected cells.
Figure 4.9: Changes induced by the Kir2.1 expression by AAV causes decrease in excitability. (A) Example of traces from Kir2.1 expressing cells and nearby uninfected cells upon rheobase injection. The amount of current injected to produced the voltage responses are shown in the bottom panel. (B) Quantification shows a significant increase in the current needed for the cell to reach the threshold (155.46 ± 36.47pA and 60 ± 5.50pA. Kir2.1 and uninfected respectively p value =0.01685).

The rheobase was measured from AAV mediated Kir2.1 over expressing cells and near by non infected cells. the results show that there is an increase in the rheobase in the Kir2.1-mCherry expressing DG granule cells (155.46 ± 36.47pA and 60 ± 5.50pA. Kir2.1 and uninfected respectively p value =0.01685).

Results in this section demonstrates that the basic electrophysiological properties between the granule cells expressing Kir2.1-mCherry via lentivirus infection and AAV infection are similar. The difference in the membrane time constant may be due to more insertion of the channel per cell in the AAV. Alternatively, the difference may be due to the smaller sampling size in the AAV group and the time constant value measured for the lentivirus infected cell may reflect the true value more accurately. Based on the similarities between the basic properties I reasoned that the adaptation would be similar and therefore used AAV infected cells for downstream experiments.
4.3.2 Electrophysiological properties of AAV-Kir2.1-mCherry infected CA1 pyramidal cells

I next turned to CA1 pyramidal cells in vivo to determine the in vivo response of pyramidal cells in response of Kir2.1 expression to assess the possible cell type differences in the mechanism of homeostatic plasticity.

I first asked if Kir2.1 over expression would show similar changes in the intrinsic properties as in the DG granule cells. Kir2.1-mCherry was expressed via AAV infection in CA1 pyramidal cells and the mouse was sacrificed 2 weeks after the virus injection. Acute slices were prepared and basic electrophysiological properties were measured in current clamp mode. The results show that the changes in membrane properties closely mimic the changes observed in DG granule cells. There was a hyperpolarisation of the resting membrane potential (-66.68 ± 1.74 mV and -72.41 ± 1.12 mV, uninfected cells and infected cells respectively) as well as decrease in input resistance (124.51 ± 7.78 MΩ and 65.04 ± 16.80 MΩ). There were also a decrease in membrane time constant (18.02 ± 1.39 ms and 8.20 ± 1.68 ms). 8 infected cells and 12 uninfected cells were sampled and all the changes were statistically significant (p < 0.05 measured by unpaired t test, precise p values are given in figure 4.10).

The rheobase for infected CA1 pyramidal cells were significantly increased when compared to the uninfected nearby neurones (68.33 ± 7.96 pA and 172.50 ± 28.77 pA, uninfected cells and infected cells respectively p value = 0.008072). This indicates that Kir2.1 manipulation can lead to changes in basic electrophysiological properties in CA1 pyramidal cells and the changes were comparable to the changes that were seen in the DG granule cells. As these changes have shown to induce suppression of activity in DG granule cells this should also cause suppression of activity in CA1 pyramidal cells also.

4.3.3 Adaptation of in vivo CA1 pyramidal cells involves synaptic changes

Previous studies demonstrate adaptation of CA1 pyramidal cells in vivo in adult in response to suppression of activity by TTX infusion (Echegoyen et al. (2007)). Also Kir2.1 has been shown in vitro to cause homeostatic adaptation in hippocampal pyramidal cells (Burrone et al. (2002)). Based on these observations I hypothesised that CA1 pyramidal cells would adapt to the suppression of activity by Kir2.1 over expression by similar mechanisms.
Figure 4.10: *Kir2.1 expression causes changes in sub threshold properties in CA1 pyramidal cells.* (A) Example trace of Kir2.1 infected and nearby uninfected cells. Current injection protocol is shown at the bottom panel and the voltage trace is shown. Region marked with blue square is shown in the right panel. (B-D) Subthreshold electrical properties of Kir2.1 expressing pyramidal cells. Kir2.1 expression resulted in significant reduction in resting membrane voltage (B), input resistance (C) and time constant (D). P values were calculated by t test and are shown on each panel (-66.68 ± 1.74 mV and -72.41 ± 1.12 mV, p=0.001156 (resting membrane potential), 124.51 ± 7.78 MΩ and 65.04 ± 16.80 MΩ, p=0.0002652 (input resistance) and 18.02 ± 1.39 ms and 8.20 ± 1.68 ms, p=9.321e-05 (time constant) Control and virus infected cells respectively)

A pair of Kir2.1 expressing cell and nearby uninfected cell were patched simultaneously as described before and both cells were clamped at -70mV. This was to minimise technical noise caused by recording conditions and connectivity in the CA1 during experiments. An example of the biocytin filled pair is shown in figure 4.12. This was done to confirm the infection profile and the cell type identity of sampled neurone. Only when the recorded pair had one infected and one non infected pyramidal cells, the data was included for the analysis. The schaffer collateral were electrically stimulated and the synaptic response from the two cells were recorded. As the pair was always from a same mouse and thought to have received very similar inputs, due to its anatomical position, paired statistics were used for analysis. The results show
Figure 4.11: *Kir2.1 expression causes increase in rheobase in CA1 pyramidal cells*. Rheobase was measured by injecting increment levels of positive current to *Kir2.1* expressing cells and nearby uninfected cells until it reached threshold level. Example traces are shown in (A) with current injected at the bottom panel. (B) The results show that the rheobase is *Kir2.1* expressing cells are significantly increased (68.33 ± 7.96 pA and 172.50 ± 28.77 pA, uninfected cells and infected cells respectively p value = 0.008072 student’s t test).

Figure 4.12: *Biocytin filling of virus infected and uninfected CA1 pyramidal cell pair*. A pair of recorded cells filled with biocytin. Biocytin is shown in green and the filled cell shows morphological characteristics of a pyramidal neurones. mCherry signal shows that the neurone noted with the white arrow has mCherry expression in the soma.
Figure 4.13: Change in electrically evoked EPSC in Kir2.1 expressing CA1 pyramidal cells. Both cells were voltage clamped at -70mV and electrically stimulated. (A) Example trace from the electrically stimulated pair. The intensity of the stimulation was altered until the synaptic response from the two cells were clearly visible. (B) The amplitude of the synaptic response were measured and plotted. The results show that there is a significant increase in the amplitude of the synaptic current in Kir2.1 expressing cells (P=0.0352, N=7, paired t test). The initial rise slop of the synaptic response is calculated and the results show that the initial slope of the Kir2.1 expressing cells are higher (B) (P=0.0204, N=7, paired t test). (C - D) The 10% to 90% rise time and full width at half maximum (FWHM) was not significantly changed (P=0.3358, P=0.2102, N=7, 10% to 90% rise time and FWHM respectively). (F) The differences between two cells in the amplitude and the 10% to 90% rise time are calculated and was divided over the sum of the two. The results shows clear deviation from 0 showing increase in the amplitude but not in the 10% to 90% rise time.

that the amplitude of the synaptic current in Kir2.1 expressing cell were significantly larger than the synaptic current measured from the uninfected cell from the same pair (-388.22 ± 64.61 pA, -272.45 ± 37.99 pA, Kir2.1 and uninfected respectively, N=7, P=0.0255, paired t test). The slope of the synaptic current was also significantly increased in response to Kir2.1 expression (-138.04 ± 31.60 pA/msec, -75.59 ± 16.64 pA/msec, Kir2.1 and uninfected respectively, N=7, P=0.0102, paired t test). However there were no significant change in 10% to 90% rise time (4.63 ± 0.80 msec, 5.71 ±
0.95, Kir2.1 and uninfected respectively, N=7, P=0.105, paired t test) indicating that the slope change is due to the change in amplitude and not the change in the temporal dynamics of the synapse. Full width at half maximum (FWHM) was calculated for each synaptic stimulation. The results show that there are no significant differences in the FWHM values in response to Kir2.1 expression (22.31 ± 3.81 msec, 23.96 ± 4.30 msec, Kir2.1 and uninfected respectively, N=7, P=0.1679, paired t test). This further indicates the change is not involving the dynamics of the synapse but are focused in the strength of the synapse (figure 4.13).

Figure 4.14: Change in spontaneous EPSC (sEPSC) in Kir2.1 expressing CA1 pyramidal cells. (A - B) Representative trace of spontaneous synaptic events. (A) Representative average of sEPSCs from Kir2.1 expressing cell (red) and uninfected cell (black). (B) Representative trace from pair of cells. (C) Analysis shows that the amplitude of each sEPSC has not changed in response to Kir2.1 expression (-22.59 ± 3.51 pA, -24.60 ± 5.60 pA, Kir2.1 and uninfected respectively, N=6, P=0.4385). (D) The frequency of the events were significantly higher in Kir2.1 expressing neurones (2.71 ± 0.68 Hz, 1.73 ± 0.58 Hz, Kir2.1 and uninfected respectively, N=6, P=0.0374). (E) Difference between the calculated values were divided by the sum of the two values and the frequency shows clear divination from 0 however the amplitude of the sEPSC does not.
What is the precise mechanism of the increase in the synaptic strength? This could include change in the postsynaptic strength (indicated by increase in the amplitude of spontaneous EPSC (sEPSC)) or/and changes in the presynapse (indicated by the change in frequency of sEPSC). Both would result in the increased amplitude of the evoked response. To determine the mechanism, post synaptic sEPSC was measured. Pairs of Kir2.1 expressing and nearby uninfected cells were clamped at -70mV and the spontaneous activity was measured for 180 seconds simultaneously from both cells (figure 4.14). The result shows that the amplitude of the sEPSC was unchanged in response to Kir2.1 expression (-22.59 ± 3.51 pA, -24.60 ± 5.60 pA, Kir2.1 and uninfected respectively, N=6, P=0.4385, paired t test) but the frequency of the sEPSC was significantly changed (2.71 ± 0.68 Hz, 1.73 ± 0.58 Hz, Kir2.1 and uninfected respectively, N=6, P=0.0374, paired t test).

This results show that the CA1 pyramidal cells in adult have adapted to the suppression of activity with increased frequency of the sEPSC events but not the amplitude of the events.

Figure 4.15: Paired pulse ratio of the Kir2.1 expressing synapses are not changed (A) Representative trace of EPSC from Kir2.1 expressing neurone and nearby uninfected neurone electrically stimulated at 0.05Hz twice. (B) Analysis shows that there are no difference between the ratio of the first EPSC and second EPSC in response to Kir2.1 expression (1.291 ± 0.144, 1.292 ± 0.170, N=4 P=0.9889).

The pair of Kir2.1 expressing and nearby uninfected neurones were stimulated at 20Hz and the ratio between the amplitude of the first and the second EPSC was calculated and compared. The intensity of the electrical signal was adjusted till the first EPSC is clearly visible. The results show that the paired pulse ratio (PPR) value does
not change in response to Kir2.1 expression in CA1 pyramidal cells.

Together these results suggests that CA1 pyramidal cells in vivo adapts to the suppression of activity through synaptic change. Synaptic changes involve increase in the frequency of sEPSCs without any changes in the amplitude of the events, suggesting an increase in the number of synaptic contacts rather than their strength. This suggests that the mechanism is similar with previous observation in vitro with Kir2.1 over expression and in vivo with TTX application (Burronne et al. (2002), Echegoyen et al. (2007)) where the authors have shown an increase in the mEPSC frequency. The authors from these studies have shown that this adaptation is of homeostatic nature.

Data presented in this chapter presents evidences of neuronal plasticity of adult neurones in vivo. Combined with the previous experiments carried out by Dr. Melanie White, Dr. Paul Dodson and Dr, Cian O’Donnell, we showed that in DG granule cells excitability is suppressed by over-expression of Kir2.1 in vivo. The change in the basic membrane properties (decreased membrane resting potential, decrease in input resistance and membrane time constant) were comparable between the lentivirus infected cells and AAV infected cells. According to simulations with a hippocampal circuit model simulations, this change in excitability should suppress synaptically driven spike output. However there was no difference between the excitability of infected and nearby non infected neurones indicating that the infected neurones have adapted to the change. This adaptation was not due to the changes in the synaptic properties of the cell but appears to be due to changes in the intrinsic properties. Also this change does not involve any changes in the soma as the rheobase was still significantly higher in the virus infected group. This would suggest that if the changes in the intrinsic property is there, this would involve a change in the intrinsic property in the dendritic region of the cell. This change is likely to be membrane voltage sensitive as the seeming change in the input output relationship is only seen at membrane voltage near the threshold. However this change needs to be further verified.

CA1 pyramidal cells showed quite a different story. When Kir2.1 was over expressed by AAV infection in CA1 pyramidal cells changes in membrane properties were similar to those observed in DG granule cells. However, in the pyramidal cells there was a increase in postsynaptic AMPA current in response to electrical stimulation to schaffer collateral indicating adaptation by synaptic changes. Investigation into sEPSC revealed that the changes are in the frequency of the events but no change
in the amplitude of the events. This also did not involve changes in the paired pulse ratio of the synapses. This suggests that the dynamics and the gain of the synapses are unchanged but the absolute number may be increased.

The experiments performed in this chapter rely on virus infection which may be different per cell. This may have different physiological consequences as seen in chapter 3 of this thesis. However the highly infected cells are likely to undergo apoptosis and have ill health. As only the healthy cells are selected during the patching experiments the extremely high infected cells would have been filtered out from this dataset. However the data suggests that the standard deviation in the virus infected group is generally larger than the nearby uninfected group suggesting that there may be a source of variability associated with different virus infection rate.

Homeostatic plasticity is defined as a maintaining of neuronal activity at a ‘set point’. This was difficult to investigate in a direct way in this system as it was difficult to measure the original excitability before the manipulation, excitability straight after manipulation then a return to original excitability over time. This was overcome by taking the nearby non infected neurone as a control group which does not show Kir2.1 over expression related changes. This would be a representative of the original state before manipulation. The infected cell was sampled 2 weeks after the injection. Due to the variability in virus infection and stereotaxic surgery it was difficult to determine the point of Kir2.1 over expression. This was overcome by measuring the changes in basic property and estimating the excitability based on the measurements through neuronal model of DG granule cells. The measurement was taken at 2 weeks after surgery which was the time point used for anatomical studies where clear mCherry reporter expression was seen. Also the excitability measured via synaptic stimulation shows no significant difference between the infected and nearby non infected neurones. This indicates that the excitability has returned to a original ‘set point’.

4.4.1 Cell type specificity in mechanism of neuronal adaptation

These results show that different types of cells may use different strategies to adapt to suppression of activity even if the mechanism of suppression is the same. There is evidence that different synapses adapt to inactivity in a different way to enhance and maintain network activity (Kim and Tsien (2008)). This work shows that even in the face of same manipulation there are synaptic distinctions which causes them to adapt differently. There are also studies where both change in intrinsic properties and the
synaptic strength are observed together (Howard et al. (2014), Tadros et al. (2014), Desai et al. (1999) and Turrigiano et al. (1998)). These results suggest that the different adaptation mechanisms can be used by different neurones to adapt to the change.

Results presented in this section provides insights into cell type specificity in plasticity even under same manipulation. Over expression of Kir2.1 in granule cell caused adaptation without any alteration in the synapses. However there were alterations in the synapse in pyramidal cells over expressing Kir2.1. The precise mechanism of adaptation was similar as preparations where TTX was used to block neuronal activity in the CA1 region or in hippocampal cell cultures (Burrone et al. (2002), Echegoyen et al. (2007)) suggesting that the adaptation by sEPSC/mEPSC frequency increase is a mature CA1 pyramidal specific mechanism. This shows that complete blocking of all action potential has similar effect as suppression of activity by changing the neuronal excitability in a sub set of cells suggesting that the change in activity in the nearby non pyramidal cells does not influence the mode of adaptation of CA1 pyramidal cells. Also there were no species specific differences (Echegoyen et al. (2007) was done in rats and this study was carried out in mouse) nor in vivo and in vitro specific differences.

4.4.2 Future work

There are some key experiments yet to be carried out. Although there is synaptic adaptation in CA1 pyramidal cells which in theory should increase excitability of the CA1 pyramidal cells this needs to be shown directly. Kir2.1 over expressing cells have adapted pairs of virus infected and un infected neurones can be patched simultaneously and electrically stimulated while the spick threshold is measured (same strategy as experiment in figure 4.4). This would give direct evidence that the synaptic change leads to normalising of the excitability after 2 weeks. The mechanism of adaptation can be probed further by measuring mEPSC which is a clearer measurement of synaptic release. Increase in the mEPSC frequency may be due to increased number of synapse which is also a known mechanism of homeostatic plasticity (Turrigiano and Nelson (2004)). Histological study can be carried out in biocytin or dye filled pyramidal cells for spine density to see if this is the case in in vivo neurones.

Another interesting aspect of homeostatic plasticity is the synapse specificity in the homeostatic adaptation. There is also a very interesting observation from Goel and Lee (2007) that the multiplicative nature of homeostasis, which is interpreted as global scaling without any synaptic specificity (Thiagarajan et al. (2005)), is lost dur-
ing development. This suggests the adaptation is synaptic specific. The results from this chapter as well as other studies (Burrone et al. (2002), Echegoyen et al. (2007)) showed scaling up of mEPSC frequency which was associated with presynaptic release probability change (Kim and Ryan (2010), Weyhersmüller et al. (2011)). CA1 receives two inputs, one CA3 through schaffer collateral which was used for evoked EPSC experiments and another from perforant pathway from the enthorinal cortex (Deng et al. (2010)). It would be informative to stimulate the perforant pathway to see the difference in the evoked response between Kir2.1 over expressing neurones and nearby un infected neurones. This would reveal if the the presynaptic changes are pathway specific shading further light into synapse specificity of the adaptation.

O’Leary et al. (2014) suggests that ion channel expression can impact the firing properties of neurones. Moreover this can lead to compensation of neuronal activity after perturbation. This suggests that, during the compensation process, there well be gene expression changes of genes where the product impacts ion conductance. DG granule cells show lack of synaptic changes but adaptation of excitability after 2 weeks. There is also evidence that the adaptation mechanism may involve change in input output relationship dependent on membrane voltage. If this mechanism is true, this may be achieved by change in the level of voltage sensitive ion channels which impact the input output relationship near the spike threshold. This expression change in ion channels is likely to involve changes in transcription. Gene expression profile can be investigated in the infected neurones and non infected neurones which would point to potential candidates involved in the adaptation of excitability in DG granule cells.
MOLECULAR CHANGES AFTER KIR2.1-MCHERRY OVER-EXPRESSION

5.1 INTRODUCTION

In previous chapters, I described a method which can be used to suppress neuronal activity in vivo and to fluorescently label the suppressed neurones. I have shown though histological experiments that this manipulation does not have any adverse effects in neurones in vivo (described in chapter 3). I have chosen DG granule cells and CA1 pyramidal cells as my model cells and shown that both neurones adapt to the suppression of activity. What is interesting is that two neurones that were studied employ different strategies for adapting to the same change. DG granule cell population does not change its synaptic properties but seems to change its input output relationship at a specific membrane potential. In contrast, CA1 pyramidal cells show synaptic changes reflected by an increase in the frequency of sEPSCs in the suppressed postsynaptic neurones (described in chapter 4).

I next sought to determine the molecular mechanisms of these changes. Studies discussed in chapter 1 of this thesis show that there are many molecular candidates which could be contributing to the control of the homeostatic plasticity. However the chain of events from the initial detection of activity to the induction of plasticity is still ambiguous. Typically, experiments have been carried out with a hypothesis driven approach where the involvement of a gene candidate was tested with knock-down or knock-in strategy. Although this strategy has been useful and allows careful investigation, it is not well suited to finding novel candidates or investigating the chain of events that may happen during plasticity. Moreover, many of the previous studies have been carried out in culture systems or in organotypic slice cultures (Sarti et al. (2013), Driscoll et al. (2013), Seeburg and Sheng (2008), Rutherford et al. (1998), Thiagarajan et al. (2002)) which may cause the cell to react with a different strategy during adaptation.

To overcome these problems I developed and characterised experimental tools to suppress neuronal activity in vivo and thereby study the subsequent adaptation of different neuronal subtypes in vivo. As these activity suppressed neurones have a
over expression of mCherry tagged Kir2.1, a pure population of mCherry positive neurones can be isolated by using FACs methods. FACs allows isolation of particles according to its size, granularity and fluorescence profile. This technique has been used in a similar setting in other studies. Lobo et al. (2006) have used this technique to isolate different cell types to compare gene expression differences. Siegert et al. (2012) and Cahoy et al. (2008) have used FACs to isolate different cell types in the retina and the forebrain also to compare cell specific gene expression. van den Berghe et al. (2013) has used this technique to investigate the molecular changes after Sip1 deletion in interneurone development.

Another technique which can achieve this is manual sorting of fluorescent cells (Sugino et al. (2006), Okaty et al. (2009)) where the cells are manually sorted with a micropipette to obtain a pure population. Laser-directed microdissection can also be used to isolate fluorescent cells for RNA extraction (Rossner et al. (2006)). Also, recently, translating ribosome affinity purification (TRAP) has been developed allowing the isolation of translating mRNAs in a specific cell type (Heiman et al. (2008), Doyle et al. (2008)). These different protocols have different pros and cons (Okaty et al. (2011)). I chose to use FACs as this demonstrates low contamination rates and high yield (Okaty et al. (2011)). By doing this it would give means of investigating molecular mechanisms after in vivo manipulations with a high sensitivity without complication of gene expression differences between different cell types.

RNA content can be analysed through different methods. Quantitative polymerase change reaction (qPCR) allows quantitative analysis of a transcript in the RNA sample. However this requires large amount of samples and it is low throughput. Alternatively, RNA can be run on a microarray chip where large number of transcripts can be analysed simultaneously. More recently, RNAseq, or next generation sequencing, has been developed for analysis of RNA samples (Mortazavi et al. (2008), Wang et al. (2009)). This allows sequencing of the complete transcriptome which also can be quantified. This approach has many advantages in comparison to qPCRs and microarrays. qPCR and microarray relies on primers and probes of specific transcripts therefore candidate selection is required before the experiment. However RNAseq relies on sequencing therefore it does not require candidate selection before the experiment. This is important for discovering potential novel candidates. RNAseq determines absolute quantity rather than the relative quantification method used by qPCRs. Also RNAseq has higher sensitivity for quantification compared to qPCR and microarrays (Wang et al. (2009)). By using RNAseq method prior candidate selection can be avoided.
This unbiased approach can be especially beneficial for mechanisms involving multiple pathways and also for novel candidate identification.

5.1.1 Experimental aim

I wanted to obtain a pure population of virus infected DG granule cells to increase the sensitivity of the experiment. Previous results from chapter 3 of this thesis suggests that the virus infected cells would also contain non granule cells near the DG. Therefore I used YFP-H line To separately label the DG granule cells with YFP (Feng et al. (2000)). I first aimed to determine if a pure population can be isolated with YFP targeted sorting. I then tested the quality of the resulting RNA samples to ensure the quality of the RNAseq experiments.

After the validation of the pure population, FACs isolation and RNAseq was performed. The resulting sequence data was analysed with previously publish sequencing data from the whole hippocampus (Stilling et al. (2014)). Then differential expression analysis was performed to determine the molecular changes after homeostatic plasticity in DG granule cells in vivo. More detailed design of the RNAseq experiment will be given in the methods section of this chapter.

Resulting gene list was then used for gene ontology (GO) analysis to determine biological functions which are important for neuronal adaptation in DG granule cells in response to suppression of activity.

5.2 Methods

5.2.1 Experimental Design

To Increase the statistical power of the study I adopted a paired design for the experiment. AAV which expressed mCherry alone was made from the same backbone and under the same promotor as AAV Kir2.1-mCherry. I used this virus to express mCherry in the DG granule cells in one hemisphere. The opposite hemisphere was injected with AAV Kir2.1-mCherry. This design has a number of advantages. There may be gene expression changes associated with AAV infection, FACs sorting or over expression of mCherry. These possible changes are all controlled for by using an AAV infected, mCherry expressing FACs sorted cells as a control group. Also, by using the same promoter equivalent genetic population can be isolated hence reduce base line noise associated with different cell types. Also as the control group and the
Figure 5.1: Experimental design for FACs sorting and RNAseq. (A) Representation of injection strategy. AAV Kir2.1-mCherry was injected unilaterally into the DG. DG on the opposite hemisphere was injected with AAV mCherry (same backbone) and used as a control for the Kir2.1-mCherry group. (B) The injection was made in YFP-H mice to isolate purer population of granule cells. YFP positive and mCherry positive population was isolated and RNA was extracted. This was amplified then subsequent cDNA was used for library preparation and RNA-seq. Reads were mapped on to the mouse genome (Mus musculus.GRCm38 release 74 from ensembl) or ERCC reference sequence. Duplicates were removed with samtools and FPKM were calculated with cufflinks2. Raw read counts were calculated by htseq-count. DEseq2 and edgeR were used for differential expression analysis. Steps in red were performed by Edinburgh genomics facility in university of Edinburgh.

For FACs method

For FACs 400µm coronal acute slice was prepared on the vibratome. The slice was recovered for 10 minutes before the DG was microdissected. Microdissected DG was treated with papain for 45 minutes at 37°C and dissociated into single cell dissociate. DAPI was added to the cell dissociate for live and dead marker and the samples were passed through a 70µm cell strainer. The components of the single cell dissociates were analysed in the FACs sorter. Cellular particles were selected based on the size and granularity. DAPI negative population was selected as live cells and from the live cells YFP fluorescence and mCherry fluorescence was assessed. RNA samples from the sorted cells were isolated. The quantity and quality of the RNA was analysed on a Bioanalyzer 2100 with a Pico chip. Concentration of the RNA and the RNA

experimental group comes from each mouse these samples were paired to increase statistical power (Ching et al. (2014)).
integrity number (RIN) was computed by Bioanalyzer specified formulas. RIN number ranges from 0 to 10, 0 being completely degrades and 10 being completely intact.

For qPCR, RNA samples were diluted accordingly and analysed with taqman qPCR method. HPRT was used as a house keeping gene. The detailed procedure of fluorescence activated cell sorting (FACS) method and subsequent RNA analysis method are presented in chapter 2 of this thesis. A systematic study by Gallego Romero et al. (2014) showed that there are changes in the RNA content as RNA is degraded. This will have an impact on the readout of the RNAseq. The study suggested that the RIN (RNA integrity number) cut of should me 6.4 to 7.9 to prevent change due to RNA degradation. To control for this I imposed a RIN cut off value at 7. Also to overcome the low RNA concentration The RNA was amplified with using Nugen RNA seq system V2. ERCC spike in was inserted with the samples to check the linearity of the amplification. The performance of Nugen RNA seq system kit has been tested with its rivalling kit, SMART mRNA amplification kit from Clontech by Adiconis et al. (2013) and shown to be similar.

5.2.3 RNAseq analysis

To control for gene expression differences associated to hemispheres, I have alternated the injection sites of the two viruses so that there are equal number of samples from either hemispheres in the group. Kir2.1-mCherry or mCherry expressing granule cells were collected with previously defined sorting gates. 8 samples (4 pairs of Kir2.1-mCherry expressing and mCherry expressing) from 4 mice were sequenced. 4 mice used for this study was from 3 different litters cancelling out possible litter specific effects. The RNA was spiked with reference RNA and amplified as described above. Two mice had Kir2.1 injection on the right hemisphere where as the other two had Kir2.1 injection on the left hemisphere. Average of 50 million reads per sample were generated. From all the reads about 40% of the reads mapped on to exons (figure 5.5 B). The mapping rate agreed with the values in the literature for amplified RNA (Adiconis et al. (2013)). The details can be found in figure 5.5.

Raw count data from HTseq count was analysed for differentially expressing genes on DEseq2 1.4.5 (Love et al. (2014)) and edgeR 3.6.8 (Robinson et al. (2010)) (Bioconductor). Two packages are both based on negative bionomial distribution model but differs in statistical strategy for estimating variance and transforming the data. The detailed performance test of the two packages are presented in (Love et al. (2014)). Both were ran with multi-factor design and analysed in pairs. For edgeR generalised liner model workflow was used with the multi factor design. For DESeq2, the built in
‘DESeq’ command was used for differential analysis with multifactor design. Cufflinks_2.1.1 were used for analysing FPKM values of each genes. This was not used for differential analysis as it did not allow paired analysis. For ERCC data I mapped the reads to the ERCC RNA spike in sequences using tophat_2.0.10 with the parameters "-r 40 –mate-std-dev 22 –solexa-quals –no-novel-junc" the used samtools_0.1.19 to remove the duplicates. I then used cufflinks_2.1.1 to calculate the FPKM for each spike in sequence. Results were visualised with either built in plot functions or custom written scripts in R or python. Scripts will be presented in the appendix of this thesis.

For gene ontology analysis, differentially expressed gene list was analysed with online gene ontology analysis tool, DAVID (Huang et al. (2009)) or GOrilla (Eden et al. (2009)).

5.3 RESULTS

5.3.1 Characterisation of YFP-H mouse line for sorting granule cells

The sensitivity of the genetic profiling experiment is greatly improved if the base line variability in gene expression can be reduced. Histological analysis presented in chapter 3 of this thesis shows that there are cells in the polymorphic layer of the DG expressing mCherry in both Kir2.1 virus injection and mCherry injection (figure 5.2). These cells are likely to be mossy cells which are the most prevalent cell type in the polymorphic layer (Amaral et al. (2007)). Sorting based just on mCherry florescence would lead to inclusion of polymorphic cells as well as granule cells in samples. As different sub-types of neuronal population has different genetic profiles (Sugino et al. (2006)), this can be a source of noise which may mask the small changes in the granule cells due to Kir2.1 over-expression.

To overcome this problem I used YFP-H mice developed by Feng et al. (2000). It has been shown that the YFP-H line has YFP expression confined to the granule cell layer of the DG (Feng et al. (2000), figure 5.2). I hypothesised that by sorting YFP positive cells, in combination with the DG microdissection, that a pure population of granule cells can be isolated. Then virus infected cells can then be isolated from the granule cell population based on the mCherry florescence from the viral trans gene expression. To test whether YFP positive cells from the YFP-H mice are all granule cells, I isolated YFP positive cells using the FACs sorter and analysed the RNA content of the YFP positive cells for cell type markers.
Figure 5.2: Injection pattern of AAV Kir2.1-mCherry in YFP-H mouse line. AAV Kir2.1-mCherry was sterotaxically injected into the DG of the YFP-H mouse. NeuN staining shows sparse cell population in the polymorphic layer. Although the infection was largely constrained to the granule cell layer there were cells in the polymorphic layer which showed clear mCherry expression (noted with yellow arrow). YFP expression in the YFP-H mouse DG was also constrained to the granule cell layer.

The scatter plots from the YFP-H mice DG FACs experiment are shown in figure 5.3. Cellular populations were determined according to the size and granularity. As DAPI positive particles indicate cells with damaged membrane allowing the entry of DAPI and binding to the nucleus, DAPI negative population was selected as the live cellular population (figure 5.3 A). DAPI signal was measured by V 450/50-A laser. From the live population the YFP expression of each particle was investigated. There were two distinct population with one showing low YFP expression and another showing high YFP expression (figure 5.3 B). YFP signal was measured by B 525/50-A laser. Two population was isolated and crossed referenced to the size versus granularity scatter plot (figure 5.3 C-D). The scatter plot shows that the two population largely overlaps in their size and granularity profile while YFP negative
Figure 5.3: Flow cytochemical characterisation of YFP positive population. (A) DAPI was added to the dissociated cells and the signal was measured by 450/50nm filter (noted with V 450/50-A labelled axis). There were DAPI negative population as well as particles showing range of DAPI positive signal. DAPI negative population was isolated as live population (Boxed). (B) From the negative population, YFP signal measurement from the B 525/50-A channel shows two populations. YFP positive population and YFP were isolated and viewed separately on forward scatter and side scatter plot. YFP positive population and YPF negative population largely overlaps in the forward scatter and side scatter plot. (C-D) The YFP negative population was more concentrated in the small particle size whereas the positive population was more concentrated at the medium sized particles.

population in more concentrated in the very small and low granular particles. The overlap may be due to the fact that not all granule cells are YFP +ve (figure 5.2). As there are YFP -ve granule cells in the sample this will overlap in size and granularity.
However the very small and less granular particles are more likely to be debris which may indicate that YFP -ve cell population may consist of non cellular particles.

One of the ways the purity can be determined is through analysing the RNA content of the sorted cells. High quality of the resulting RNA can be an indication of healthy pure cell population free from debris or dead cell contamination. To determine the content of the sort I isolated different numbers of YFP positive and YFP negative population by FACS. I then isolated RNA from the sorted cells and analysed the RNA with a bioanalyzer 2100.

<table>
<thead>
<tr>
<th>Cell number</th>
<th>YFP positive population</th>
<th>YFP negative population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc (pg/ul)</td>
<td>RIN number</td>
</tr>
<tr>
<td>500 cells</td>
<td>103</td>
<td>7.1</td>
</tr>
<tr>
<td>1000 cells</td>
<td>101</td>
<td>7.6</td>
</tr>
<tr>
<td>2000 cells</td>
<td>265</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Table 5.1: Characterisation of RNA from FACS sample. Set number of YFP positive or negative particles were isolated from with FACS and RNA was isolated. RNA samples were ran with Pico-chip on bioanalyzer. YFP positive population shows consistent RNA concentration over 100pg/ul. RIN numbers for all samples were above 7. For YFP negative population there were no reliably detectable amounts of RNA even with 2000 cells. RIN number was also very low.

The results from the bioanalyzer show that, in the YFP positive population, intact RNA can be isolated even from as little as 500 cells. The RIN numbers from the RNA samples were also within the cutoff value (table 5.1). RIN of 7 or above was used as a cutoff for this study. However, the RNA concentration and the RIN number for 500 cells were not consistent. Some of the sorts with 500 cells showed no detectable RNA. This may be due to the unreliable RNA extraction efficiency at the low amount of starting material. The concentration and the RIN values from 1000 cells were consistent between different sorting sessions. The minimum number of cell number for all the sorts were set to 1000 cells before proceeding to RNA extractions. In YFP negative population there was no detectable RNA even at 2000 sorted cells (table 5.1). This indicates that the cell gate which is just based on the size and the granularity of the particles is not a pure population of cells but contains other non cellular particles which resembles a cell in terms of size and granularity. The observation that the YFP -ve population is more concentrated at the small and low granular particles supports this interpretation (figure 5.3). Pooled data from all the sorts show that there is a liner relationship between the number of YFP +ve cell sorted and the RNA concentration (Pearson’s product-moment correlation, Cor = 0.6741696, p = 0.002999). The low
correlation value may also be due to the impurities in the cell gate. Contamination of non cellular particles will reduce the number of cells in the sample thus compromising the correlation of RNA concentration and cell number read out from the FACs sorter. However, the impurities did not compromise the RNA quality.

Figure 5.4: Genetic characterisation of YFP positive population. 1000 particle of the YFP positive population was isolated and the RNA was isolated. Expression levels of various cell type specific marker was measured with qPCR and was compared with RNA samples from micro-dissected DG and RNA sample from the whole hippocampus. YFP sorted samples consistently shows similar levels of MCM6 (DG granule cell marker) and very low levels of NeuroD6 (CA1 pyramidal cell marker), Gfap (astrocyte marker) and Gad2 (interneurone marker).

With the quality of RNA established, I next asked if the YFP positive population is indeed a pure granule cell population. To answer this question, I analysed cell marker expression level from the RNA isolated from YFP +ve cells. As a control group I have used RNA isolated from whole hippocampus and RNA isolated from micro dissected DG. I chose to assess the levels of MCM6 (DG granule cell marker), NeuroD6 (pyramidal cell marker), GFAP (astrocytic marker) and GAD2 (interneurone marker). These cells are all found in the hippocampal region therefore may contaminate the sample. qPCR results from the samples from micro-dissected DG shows enrichment of MCM6, and slight depletion of NeuroD6, GFAP and GAD2. However when the YFP +ve cells were sorted there was a further enrichment of MCM6 and a near complete depletion of NeuroD6, GFAP and GAD2 (2 way ANOVA P<0.0001, post hoc shows significant difference only in the MCM6, N=3) which indicates that YFP +ve cells are a pure population of granule cells without any contamination from pyramidal cells, astrocytes or interneurons.
These results together show that by using YFP-H mouse line, DG granule cells can be isolated by YFP fluorescence. The cell gate set in the FACs sorter shows indications of non cellular impurities however, by using an additional YFP dependent sorting gate I was able to isolate cellular populations resulting in intact RNA from only a small number of particles. qPCR further reveals that the YFP +ve particles express high levels of DG granule cell marker and low or undetectable levels of astrocytes, pyramidal cells nor interneuron marker which are abundant in the hippocampus. Thus it can be concluded that the YFP positive population are a pure granule cell population.

5.3.2 Validation of RNA-seq results

Previous results show that the sorted cells are pure granule cell population and the subsequent RNA is of good quality. RNA from Kir2.1-mCherry or mCherry expressing granule cells were sequenced. The sequences generated were mapped to the reference genome and the FPKM values were determined by cufflinks. RNAseq results were investigated for further confirmation of the specificity of the sort.

I first investigated the expression of several genes in the allen brain atlas for their expression in the DG granule cell layer. Previously known granule cell marker MCM6, Prox1 and Calb1 was expressed highly in the DG granule cell layer. Calb1 was also highly expressed in the CA1 region. POMC, a newborn granule cell marker (Overstreet et al. (2004)), was undetectable in the image. GAD2, an interneuron marker, and GFAP, an astrocyte marker, was expressed throughout the hippocampus but did not show the tight expression pattern of the granule cell markers. NeuroD6 (pyramidal cell marker) showed tight expression in the CA1, CA2 and CA3. I also assessed the expression pattern of CD68 (microglial marker). There were small amount of expression scattered in the hippocampus (figure 5.5 D). These observations show that the cellular marker gene chosen for FACs validation has expected expression pattern, further reconfirming the validity of the marker gene.

Sequenced YFP and mCherry double positive cells were assessed for marker genes used above. To do this I assessed the FPKM levels for the abundance of the gene in the sample. The samples were abundant in granule cell markers (MCM6 17.73, Prox1 323.48, Calb1 106.40). In contrast, POMC GAD2 (interneuron marker), NeuroD6 (Pyramidal cell marker), CD68 (microglial marker) and GFAP(astrocyte marker) were absent or at very low levels (0.87, 1.68, 0.47, 7.50, 0.80, respectively, figure 5.5 C). FPKM values agrees well with the in situ hybridisation data from Allen brain atlas.
Figure 5.5: Sample collection and characterisation of RNA-seq reads. (A) Kir2.1-mCherry or mCherry virus was injected to either hemisphere in the same mouse. (B) Kir2.1-mCherry and mCherry injected DG was collected from 4 mice from 3 different litters of mice. Injected hemisphere were altered between mice. All samples contained at least 1000 sorted cells subsequent RNA samples all showed RIN > 7. RNA samples were SPIA amplified and sequenced on illumina Miseq sequencer. On average, 50 million reads per sample were generated. (C - D) FPKM values for cell specific markers are analysed. As seen in qPCR results from YFP sorted cells there were high levels of granule cell specific markers (MCM6, Prox1, Calb1). There were no or low levels of newborn granule cell marker (POMC), interneuron marker (GAD2), CA1 pyramidal cell marker (NeuroD6), microglial marker (CD68) and astrocyte marker (GFAP). (D) in situ images from allen brain atlas (Coronal plane where available) for granule cell specific gene show expression constrained to granule cell layer where as other cell type specific markers shows little or no expression the granule cell layer. (E) FPKM values of spick in control was plotted against the input concentration. Average values were marked with + for each spike RNA. FPKM and the input concentration shows a liner relationship.

This shows, with the qPCR results from YFP sorting, that the sorted population is granule cell specific and even with RNAseq non granule cell markers
are detected at a very low level.

The generated reads were also mapped on to the ERCC spike in control reference sequences using bowtie and the levels of reference RNA was assessed with FPKM values calculated by cufflinks. This would assess the linearity of the amplification and the library preparation stage. Out of 92 standard RNA sequences inserted into the sample, 7 to 22 standard RNA strands were detected in the sequenced samples. The detected control RNA strands were the strands with highest input quantities and within the detected reference RNA strands there was a positive liner relationship between the input concentration and the FPKM value indicating that the read generated from the sequencing experiment is an accurate representation of the RNA levels in the original RNA sample (figure 5.5 E). The low number of detected RNA spike in sequences is likely to be due to the low input level as the higher concentration input strands were detected. These results suggests that the gene counts generated from the amplified RNA are linearly related to the original transcript levels in the RNA sample.

One other way to validate the specificity of sorting is to compare the read counts from granule cells with the published RNAseq data from whole hippocampus. Where cell marker gene expression levels in figure 5.5 shows high levels of granule cell specific genes and low levels of non-granule cell specific genes this is not a comparative measure. However by comparing the read count values from sorted granule cells to read count values from the whole hippocampus, a quantitative measure of the enrichment of granule cell specific genes and depletion of non-granule cell specific gene can be determined for further verification. Furthermore this could reveal previously unknown granule cell specific genes.

The data used for verification was taken from Stilling et al. (2014) where the authors used RNAseq to investigate the genetic changes associated with ageing. Data from Stilling et al. (2014) was from a mouse line with the same background genetics as YFP-H used in this study (C57BL/6J). In order to rule out the differences caused by Kir2.1 over expression, only the reads from mCherry expressing granule cell group were used.

The read count data from the two dataset was extracted and differential gene expression was analysed with DESeq2 package. Using adjusted P value < 0.05 as criteria 5409 genes were shown as DG enriched and 5410 genes were shown as DG depleted. The log fold change ranges from 0.23 to 11.16 for enriched genes and 0.24 to 14.45
Figure 5.6: *DG specific gene expression pattern* Previously published data from the whole hippocampus was compared with mCherry expressing DG granule cells. (A) Differential expression analysis revealed 10819 gene to be differentially expressed using adjusted p < 0.05 as criteria. 5409 were upregulated with log fold change (LFC) ranging from 0.23 to 11.16 and 5410 were downregulated with LFC ranging from 0.24 to 14.45. (B) LFC values were plotted against the mean expression value for all genes. All significantly differential genes are plotted in red. (C) LFC of the conventional cell marker genes were plotted. Granule cell expressed genes (in red) were all upregulated whereas a common housekeeping gene, HPRT (in green) showed very little depletion. In genes known to be absent in granule cells (HCN1, NeuroD6, GFAP and GAD2, in blue) were all down regulated. (D) Gene ontology analysis reveals various cellular components which are enriched. The list is focused on neuronal parts.

for depleted genes (figure 5.6 A-B). Log fold changes of marker genes were investigated. All the granule cell markers examined (MCM6, Calb1, and Prox1) were shown
to be enriched. HPRT, a common house keeping gene and the house keeping gene of choice in this study, had very little log fold change and was not differentially expressed. HCN1, which was previously shown to be expressed at lower levels in granule cell compared to CA1-3 cell population (Bender et al. (2003)), was shown to be significantly depleted in the granule cells. Also cell markers of different neuronal types (pyramidal cells, astrocytes and interneurons) were also significantly depleted.

List of all 10819 differentially expressed genes were analysed for enriched GO terms using DAVID (david.abcc.ncifcrf.gov, Huang et al. (2009)) to assess in which GO terms the changes were concentrated. Full results of this analysis will be presented in the appendix. As a representative example, GO terms under cellular components are presented in figure 5.6 (D). Axon initial segment was the GO term which was most enriched for followed by terms related to ribosomal parts. The rest of the list was focused on neuronal cell parts such as terminal boutons, axon parts and postsynaptic density.

Together these results shows that the read count generated from the sorted granule cells represents the granule cell transcriptome.

5.3.3 RNA-seq reveals gene candidates involved in neuronal adaptation in response to Kir2.1 overexpression

The read count values from the Kir2.1 over-expressing granule cells and mCherry expressing cells were analysed for differentially expressing genes using DESeq2 package (Love et al. (2014)) and edgeR package (Robinson et al. (2010)) to reveal gene expression changes during neuronal adaptation.

DESeq2 was ran with built in function for differential expression analysis. MA plot was used to visualise the mean expression level across the sample and the log fold change value calculated from DESeq2. MA plot was plotted with each grey dots representing each gene with differentially expressing genes plotted in red (adjusted p < 0.05, figure 5.7 A). There were 101 genes differentially expressed using adjusted p < 0.05 as criteria, and 210 genes using adjust p value < 0.1 as criteria for differential expression (figure 5.7 A). Full list of differentially expressed gene is provided in the appendix of this thesis. Principle component analysis (PCA) was used to assess variability between the samples. The PCA plot reveals that mCherry expressing granule cells from mouse 1 and Kir2.1 expressing granule cells from mouse 2 was largely different from rest of the samples. However the aspect of this difference was
5.3 RESULTS

Figure 5.7: Expression analysis with DEseq2 package (A) MA plot of all genes with values calculated by DEseq2. log2 fold change is plotted against the means of normalised counts. Each point represents each gene and differentially expressed genes are noted in red. (B) PCA analysis of count data by DEseq2 package. PCA plot shows that there are no major differences between samples with exception of 2 samples. mCherry sample from mouse 1 has difference in the PC2 component and Kir2.1 sample from mouse 2 has difference in the PC1 component when compared to the rest of the samples.

not the same in the two samples which can be seen by the different dimension in the diversion from the rest of the samples (figure 5.7 B).

To control for any package specific effect and also to probe the RNAseq results further, EdgeR was ran also with built in functions using the same read count data. MA plot was plotted with log2 count per million data and log fold change values calculated by the edgeR package (figure 5.8 A). As edgeR package and DESeq2 package differs in the method of data transformation the shape of MA plot appears different with the main difference at the low expressing genes (figure 5.7 A and figure 5.8 A). There were 23 differentially expressing genes using criteria of false discovery rate (FDR), an equivalent of adjusted p value in DESeq2, of < 0.1 which are plotted in red in the MA plot (figure 5.8 A). There were 19 genes with FDR of < 0.05. Multi-dimension scaling plot (MDS plot) function is provided in edgeR package as an equivalent of PCA plot in DESeq2 package. MDS plot of all the samples also reveals that mCherry expressing granule cells from mouse 1 and Kir2.1 expressing granule cells from mouse 2 is largely different from other samples in the data set (figure 5.8 B).
Figure 5.8: Differential expression analysis with edgeR package. Count data was analysed with edgeR. (A) the equivalent of MA plot in DEseq2 is shown. There are differences in the general shape. The difference is mainly on the low expressing genes. Blue line denotes log fold change of 1 or -1. (B) PCA plot (MDS plot) was plotted with the edgeR built in function. mCherry1 and Kir2 are shown to be distant from the rest of the samples.

Figure 5.9: Difference between edgeR and DESeq2. (A) Log fold change values calculated for each gene in two different packages were plotted. There was a general linear trend between the log fold change values calculated from the two packages. Differentially expressing genes from DESeq2 were plotted in red and genes which appears in both list were plotted in black. Differentially expressing genes from only edgeR was plotted in green (B)There were 210 differentially expressing genes in DESeq2 list and 23 on edgeR list. All the genes from edgeR list was present in the DESeq2 list.

To further reveal the difference between the two packages, results from the two packages were compared. The log fold change values from each gene calculated from the two packages were plotted (figure 5.9 A). The plot reveals that the log fold change values from the two packages have a liner relationship. However, there were
some variations and there was a general tendency for edgeR to estimate the log fold change value larger than DESeq2. This can be seen from the shape of the plot in figure 5.9. Also, the average log fold change values of all the positively changed genes and negatively changed genes were larger in edgeR than the values from DESeq2 (negative LFC -1.172, -0.73. Positive LFC 1.045, 0.63. edgeR and DESeq2 respectively). There were 210 differentially expressed genes in the DESeq2 list and 23 genes in the edgeR. All of the genes from the edgeR list also appear in the DESeq2 list (figure 5.9 B). Log fold change values calculated by DESeq2 and edgeR were more similar in the differentially expressed genes compared to all genes.

5.3.4 Functional changes associated with Kir2.1 over expression

The list of differentially expressed genes can give information on the involvement of individual genes. However, all the genes in the list can be grouped according to gene ontology (GO) groups to reveal biological categories which are associate with the gene list.

GO analysis with online tools (DAVID Huang et al. (2009) and GOrilla Eden et al. (2009)), on the 210 genes that were differentially expressed in DESeq2 package did not reveal any enriched gene categories. However, the physiology data presented in chapter 4 of this thesis suggests a change in membrane conductance at a certain membrane potential. This is likely to be due to changes in ion transporting causing a change in the activity in voltage dependent ion transporter as it will give the combination of membrane potential selectivity and change in input output relationship. To investigate the genes that are involved in ion transporting more closely, all the genes that fall under gene ontology category ‘transporter activity’ (GO:0005215) were highlighted in the dataset (figure 5.10 A). There were 14 transporter activity related genes which were differentially expressed in the DESeq2 list of which 9 had FDR < 0.05. There were 3 genes which were differentially expressed also in the edgeR list.

To assess the validity of the differential expression packages candidate genes with different statistical significance were selected and the expression levels were measured with qPCR. Genes were selected so that a range of adjusted p values and log fold change values can be tested. The DESeq2 calculated fold change values and the adjusted p values are shown in figure 5.11 A. Ptprf, plekhh1 and nrgn were also tested but those genes were undetectable with qPCR. 4 pairs of Kir2.1 and mCherry
Figure 5.10: Analysis of expression changes of transporter related genes in response to Kir2.1 expression. (A) MA plot from DESeq2 and edgeR. Genes which fall under transporter activity (GO:0005215) are highlighted in red with differentially expressed genes in green (FDR < 0.1). There were 14 genes which were differentially expressed in the DESeq2 package and 3 genes in edgeR package. (B) Log fold change values of the genes from both list (excluding Kir2.1) are given in green. All the genes which had FDR < 0.05 in the DESeq2 list are shown in red and the genes which had FDR < 0.1 are shown in blue.

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<th>Gene</th>
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<th>Fold enrichment</th>
<th>Adjusted P</th>
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<td>1.54588</td>
<td>0.0352758</td>
</tr>
<tr>
<td>Mal</td>
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<td>0.111991</td>
<td>1.74e-05</td>
</tr>
</tbody>
</table>

Figure 5.11: qPCR validation of RNAseq results. (A) selection of genes from the differentially expressed gene list were selected and the expression levels were confirmed with qPCR. Selected genes had a range of adjusted p values and and log fold changes calculated by DESeq2. Enrichment value was also calculated from the log fold change values. (B) 4 Pairs of Kir and mCherry expressing granule cells were used for RNA extraction and qPCR. Measured expression levels were normalised to HPRT and the expression level from Kir2.1 expressing granule cells were normalised to the expression level in the paired mCherry granule cells. Expression level from RNA seq was calculated from the raw read count. As with qPCR data the counts were normalised to the level of HPRT and the expression level from mCherry. There were no significant differences between the change in expression level measured by qPCR and RNAseq (Two way ANOVA p=0.9637).
expressing granule cells were sorted and the RNA was extracted. Extracted RNA was analysed with taqman qPCR method. Measured gene expression value from qPCR was normalised to HPRT level. The expression level of from Kir2.1 expressing granule cells were then normalised to the expression level from the mCherry pair to calculate the fold enrichment in response to Kir2.1. To minimise the differences caused by the normalisation process in RNAseq results the raw count data was normalised in the same way as the qPCR data. The number of counts was normalised to the number of counts for HPRT in the sample and normalised to the paired mCherry expressing sample from the same brain. ANOVA shows no significant difference (p=0.9637) between the fold enrichment measured from qPCR and RNAseq data. Post-hoc analysis shows that there is a significant difference on the Nos1 gene levels. This may be due to the high adjusted p levels of Nos1 (adjusted p = 0.074).

These results show that there are gene expression changes associated with expression of Kir2.1mCherry. GO analysis did not reveal any enriched GO categories but individual assessment of the candidates reveals that there are some gene candidates which have been linked with homeostatic plasticity as well as some novel candidates.

5.3.5 Analysis of individual samples to for assessment of variation

Differential expression analysis reveals that mCherry sample from mouse 1 (mCherry1) and Kir2.1 over expressing sample from mouse 2 (Kir2) may be outliers. To investigate this possibility further the calculated expression values from DESeq2 for individual samples were analysed. Regularised log transformed value was used throughout this analysis to reduce variation in the RNAseq data.

Gene expression levels from Kir2 and Kir2.1 over expressing sample from mouse 3 (Kir3) were plotted against each other. The plot reveals that there is a group of genes in Kir2 which were over expressed (figure 5.12 A). Such differences were absent in the same plot with Kir3 and Kir4 which were in similar positions in the PCA plot. Same plot from mCherry 1 with other mCherry samples also reveals a similar but different situation where there were genes which was both up regulated and down regulated where as the comparison between mCherry 3 and mCherry 4 reveals a liner relationship without much variation (figure 5.12 A).

Genetic analysis of the individual mouse was carried out by combining count from Kir2.1 samples and mCherry samples. This was done by redesigning the formula in DESeq2 so that the mouse number was set as the experimentally relevant term. This
will allow testing of differentially expressing genes in different mouse rather than the differentially expressing genes between the mCherry over expressing and Kir2.1 over expressing cells. In the mouse 2 there was massive down regulation of α synuclein which suggested that mouse 2 lacked α synuclein in the genome. What is interesting is that this α synuclein deletion had no apparent effect in the mCherry expressing cell seen in the plots. The genealogy of the mice used in the study is given in (figure 5.12 B). Genetic analysis of mouse 1 reveals no apparent mutations in the genome of the genome however in the plot where the sorted cell number and the resulting RNA concentration mCherry 1 shows a dramatic diversion from the liner trend (figure 5.12 B). The results above suggests that Kir2 and mCherry 1 is indeed an outlier. The reason for this may be due to α synuclein deletion in mouse 2 causing different response after Kir2.1 over expression. Only detected difference in mCherry 1 was the relationship between cell number and RNA concentration. The RNA quality and the sorting criteria were for mCherry1 was similar to other sample.

The results above and the PCA plots suggests that Kir2 and mCherry 1 is an outlier. To reduce the variability caused by these outliers, mCherry1 and Kir2 was removed from the sample set and DESeq2 was used for differential expression analysis. The samples were tested with a unpaired design. Analysis revealed 145 genes which were differentially expressed (P adjusted < 0.1). The differentially expressed gene list from all samples (all sample) and with the outliers removed (subset) were compared. There were 75 genes which were in both lists and 70 genes exclusive to subset. Log fold change values of each genes in the two lists were plotted. This reveals that the genes which were common to both lists and genes in the all sample list show similar log fold change levels between all sample analysis and subset analysis. Genes which were exclusive to all sample analysis were genes with lower log fold change values. However genes that were exclusive to subset analysis had a tendency to have higher log fold change value in the sub sample analysis (figure 5.13 A). This suggest that the difference between the two analysis is mainly due to change in statistical power associated with higher sample number and paired design.

5.4 DISCUSSION

Hypothesis driven experiments identified a number of genes which are shown to change during homeostatic plasticity (Turrigiano (2012), Turrigiano (2011)). However these work has largely been carried out in neuronal culture systems. Also as the experimenter is investigating one hypothesised candidate molecule at a time in what
might be a complicated process involving interaction of many molecular pathways, this approach may not reveal a full complement of gene expression changes contributing to homeostatic plasticity. Here, I used virus infection to change neuronal activity and induce neuronal plasticity in DG granule cells *in vivo*. The mechanism of the adaptation is described in chapter 3 of this thesis. I then used FACs and RNAseq to isolate the transcriptome of adapted cell and analyse all the content in a quantifiable way. This allows molecular changes associated with neuronal plasticity *in vivo* to be studied in an unbiased way.

5.4.1 *Isolation of pure granule cell population by FACs*

To ensure the sensitivity of the RNAseq experiment, a pure cell population needs to be isolated. There are mounting evidences that there are cell type differences in molecular expression (Lobo et al. (2006), Siegert et al. (2012) and Cahoy et al. (2008)).
Figure 5.13: Comparison between differentially expressing genes in all samples and sub set of samples. Differential expression analysis with all samples and the subset of samples (except Kir2 and mCherry) were compared to assess the possible changes (A) Log fold change values calculated for each gene in two different groups were plotted against each other. Genes that were shown to be differentially expression on both list is plotted in black, genes that were specific to all sample list were plotted in green and the genes that were specific to subset list were plotted in red. (C) MA plots from the two groups are shown.

Mixed cell population of interest will increase variability caused by gene expression differences between cell types and may mask the small molecular changes associated with the plasticity. In addition, minimisation contamination of debris, which can introduce RNA from other cell type and RNase from apoptotic cells, must be achieved.

To isolate a pure granule cell population YFP expression in YFP-H mouse line was used (Feng et al. (2000)). By adopting this strategy there will be mCherry positive cells (virus infected cells) in the granule cell layer lost due to the absence of YFP fluorescence as there are YFP -ve cells in the granule cell layer that are mCherry +ve. This may lead to lower numbers of cells thus reducing the amount of RNA or selection of subpopulation of granule cells which may misrepresent the molecular change. However there is little reason to suggest that the YFP positive cells have physiological distinction from the rest of the granule cell population and there are sufficient number of cells to isolate enough RNA for sequencing. Furthermore as the control population was also from a YFP positive population, the detected differences would all be related to the homeostatic plasticity. Therefore I reasoned that the benefits of obtaining a pure population of granule cells are greater then the possible limitations. RNA from YFP positive cells shows enriched marker of DG granule cells and de-
pletion of interneuron markers, astrocytic markers and CA1 pyramidal cell markers. The YFP-H mouse line has YFP expression in the CA1 pyramidal cells but this was removed by the micro dissection of the DG and also confirmed by the absence of CA1 pyramidal cell markers.

Subsequent analysis of RNA isolated from the sorted YFP positive population revealed detectable RNA concentration with high RIN score (7 or above) with 1000 sorted YFP positive DG granule cells. Degradation of RNA can cause changes in the level of different transcripts at a different rate (Gallego Romero et al. (2014)). Therefore, it is important that the RNA from the sorted cells are at a high quality to ensure that the RNA levels are a true representation of the transcript levels in the cell. The results also shows that a high RIN score RNA can only be isolated from the YFP positive cells. This suggest that the cell gate based on the size and granularity has contamination of non cellular particles which compromises the concentration and the RIN score of the RNA sample. This indicates a further benefit of sorting fluorescently labelled cell populations.

5.4.2 Paired design to increase statistical power

The sorting experiment was designed to minimise technical noise. Experience of can change gene expression such as Arc or c-fos to induce synaptic plasticity (Bramham et al. (2010), Flavell and Greenberg (2008)). As the experience of the experimental mice were not in anyway controlled, this may introduce some noise in the transcripts between different mice. Another issue is that the activity suppression was induced by viral infection. This may also induce some molecular changes that are related to the AAV infection and not the change in activity caused by the expression of the trans gene. Also mCherry is expressed with the Kir2.1 channel. Although there is little evidence to suggest that mCherry will cause changes in gene expression this is difficult to fully rule out.

To control for all above factors, experiment was set up so that the control cells are also infected with AAV which expresses mCherry. This controls for potential gene expression changes by AAV infection and mCherry expression. Furthermore, Kir2.1-mCherry and mCherry virus was injected to each hemisphere in a mouse. Granule cells from each hemisphere were isolated to make one sample. This allows control of potential gene expression differences due to different experience that the mouse might have had during its life course as both groups are from the same mice. Also,
this allows a paired approach in the analysis which has also been shown to increase the statistical power of the study (Ching et al. (2014)).

There are concerns with possible mixing of samples during experiment and inability of unequivocally distinguishing the control group and the Kir2.1 over-expressing experimental group due to the use of same fluorophore. However this was confirmed after the sequencing run by checking the over-expression of Kir2.1 in the experimental group. Also there are concerns that there may be a network effect due to the silencing of subset of granule cells in the Kir2.1 expressing but not in the control group. The can potentially lead to network induced changes as well as the cell autonomous effects which is subject to investigation in this study.

5.4.3 Validation of RNaseq experiment

Analysis of the RNAseq results shows that the samples are from a pure population of DG granule cells as there are high levels high levels of granule cell markers and low levels of other cell type markers. Importantly the ERCC RNA spike in which was inserted before the amplification process showed a liner relationship in the RNAseq read out. However, only the highest input concentration was detected in the RNAseq sample. This may be due to the insufficient level of RNA spike in in the original sample. Despite of this, as the detected spike in levels and initial input concentration had a liner relationship, I concluded that the amplification process is liner and that the transcript level detection in the RNAseq reads represents the level of transcripts in the original RNA sample.

Further verification was performed by performing differential expression analysis comparing the mCherry expressing granule cells to previously published RNAseq results from the whole hippocampus (Stilling et al. (2014)). There are some differences and similarities between the RNA preparation and sequencing between the study I carried out and the study Stilling et al. (2014) carried out which may have an impact on the transcript of the cell. Main similarity is the genetic background of the mice. The YFP-H mice used in this study was kept on the C57BL/6J background and the mice used for Stilling et al. (2014) was also C57BL/J6. As genetic background of the mice will certainly result in the differences in gene expression, having the same genetic background for comparison is a major advantage. Some other similarities include library prep methods and sequencing platform. The main differences between the two samples are the amplification process and the age difference between the samples.
Sequence data from the control group (RNA from whole hippocampus from 3 month old) were taken for comparison between the data from sorted granule cells from 7 week old mice. As Stilling et al. (2014) suggests that there are differences in gene expression dependent on age, this may be a source differences in gene expression. Also the samples from granule cells were amplified where as the data from Stilling et al. (2014) was not. This may also introduce another source of variation. However the differences caused by age and amplification process may be subtle in comparison to the gene expression differences caused by different cell population or different genetic background of the mice.

I reasoned that this would reveal DG granule cell specific genes as well as further verifying that the identified genes represents genuine biological transcripts. The differential expression analysis reveals that there are substantial differences in the gene expression profiles. As expected there was an enrichment of DG granule cell markers and depletion of other cell type markers. Gene ontology analysis of the differentially expressing genes show that the difference is concentrated on the neuronal parts.

5.4.4 Gene candidates for Kir2.1 over expression induced neuronal plasticity in DG granule cells

Differential expression analysis has detected 101 differentially expressed genes (P adjusted < 0.05, 210 genes with P adjusted < 0.1) associated with Kir2.1 over-expression induced plasticity in DG granule cells in vivo. 8 (13 with p adjusted < 0.1) of these were associated with GO term 'transporter activity' which is potentially important considering the physiological mechanism of adaptation in DG granule cells (see chapter 5 of this thesis for details). Ptgds (prostaglandin synthase D2) was expressed at a lower level during homeostatic plasticity. Prostaglandin E1 has been linked with synaptic plasticity (Sang et al. (2005), Akaneya (2007)). Prostaglandin D2 has also been suggested to act with pre synaptic NMDA receptors to increase Ca²⁺ in the synaptosomes (Petrova et al. (2007)) and astrocytic Ca²⁺ dependent glutamate release (Bezzi et al. (1998)).

Interestingly there were changes in the level of 4 solute carrier family proteins (Slc17a6, Slc24a5, Slc6a18 and Slc6a1). Slc17a6 encodes VGlut2 protein which is involved in vesicle glutamate transport and is expressed at low levels in the DG (Reimer (2013)) and Slc24a5 encodes NCKX5 which is a Na⁺/(Ca²⁺-K⁺) exchanger (Schnetkamp (2013)). Slc6a1 is a transporter of GABA and the substrate of Slc6a18 are yet unknown.
but the mutation is linked to myocardial infarction (Pramod et al. (2013)). As these solute carriers are involved in transporting ions and substrates they are a possible candidate for the change in the excitability near the action potential threshold. This needs to be confirmed with more careful experiments manipulating each component.

Itp2 (inositol-1,4,5-trisphosphate receptor 2) is involved in Ca\textsuperscript{2+} buffering from the endoplasmic reticulum (Mikoshiba (2006)) and is also involved in synaptic communication in astrocytes (Di Castro et al. (2011)). Orai1 (Calcium release-activated calcium channel 1) is also involved in Ca\textsuperscript{2+} buffering and is shown to be important for neuronal function (Venkiteswaran and Hasan (2009)). Two genes were significantly down regulated in response to Kir2.1 infection. This would lead to depletion of intracellular Ca\textsuperscript{2+} given the function of two genes (Hewavitharan et al. (2007)). Why the cell would react to suppression of activity, which would lead to the lowering of intracellular Ca\textsuperscript{2+}, by making changes that will lead to further depletion is not know. Moreover, whether this change, in combination with others, do indeed lead to further depletion of Ca\textsuperscript{2+} is still unclear.

Apod has shown to regulate the transcriptional profile of neurones (Sanchez et al. (2015)). Kcnq3 is a subunit of ‘m channel’ which gives m current. An increase in the m current has been linked to decreased excitability in the neurones (Wang (1998)).

There were no obvious links between all the transporter activity genes in the differentially expressed gene list. Some, such as Kcnq3 and itpr2, were regulated in an opposite direction from the expected regulation. However, the combination of all the changes may work to enhance the excitability. There were genes in the list that are involved with Ca\textsuperscript{2+} buffering within the endoplasmic reticulum as well as genes from the solute carrier family involved in different aspects. This list provides a candidate list which one can use in a hypothesis driven study to identify the precise molecular mechanism behind Kir2.1 induced change in intrinsic properties of DG granule cells.

Apart from genes linked to transporter activity there are changes in the level of other genes. Amongst the list CAMK4 (see section 1.2.3.4 of this thesis, Ibata et al. (2008)) and NOS1 (see section 1.2.3.7 of this thesis Steinert et al. (2011)), were differentially expressed as predicted from previous work (for details of the literature refer to chapter 1 of this thesis). However, these genes have previously been shown to be involved in mainly synaptic scaling. As previous experiments have all been carried out in vitro the molecules may have a different function. At least in DG granule cells
the combination of the changes in gene expression may be acting in a way to alter the intrinsic properties rather than the synaptic strength.

5.4.5 Future work

An obvious future direction is to sort CA1 pyramidal cells to determine the molecular mechanism of homeostatic plasticity by increase in the frequency of mEPSC. Physiological changes seen in the CA1 pyramidal cell suggests increase in the number of synapses following over expression expression of Kir2.1. Analysis of transcriptome of CA1 pyramidal cells will reveal post synaptic gene changes associated with the increased frequency sEPSC.

On going work in the Nolan lab suggests that YFP-H line is inadequate for CA1 sorting as the YFP expression in the CA1 is too sparse resulting in insufficient RNA concentration. To overcome this we have developed a cre dependent Kir2.1-mCherry and mCherry virus by using flex switch system (Murray et al. (2011)). Flex switch system allows expression of transgene only in cre positive neurones. By putting the flexed transgene under a ubiquitous promoter combined with cell type specific expression of cre, cell type specific transgene expression can be achieved. On going work also suggest that by using a cre line under control of Sim1 promoter (GENSAT project), around 500 virus infected cells can be isolated. The qPCR and bioanalyzer results suggest that the population is a pure population of pyramidal cells and the RNA is of good quality.

Work from Echegoyen et al. (2007) suggests that there is a developmental difference in the mechanism of homeostatic plasticity. Adult CA1 pyramidal cells show adaptation by increasing mEPSC frequency while juvenile CA1 pyramidal cells adapt by increasing mEPSC amplitude. As Kir2.1 causes same adaptation in the adult pyramidal cells it is possible that Kir2.1 will also cause the same adaptation in juvenile CA1 pyramidal cells. This classic synaptic scaling mechanism has been well characterised in in vitro systems (Turrigiano et al. (1998), for reviews see Turrigiano (2008), Turrigiano (2012)). Same experimental protocol can be repeated in juvenile mouse to confirm the mechanism of adaptation and subsequent molecular profiling of the cells to potentially reveal transcriptional changes in homeostatic plasticity in vivo.
GENERAL DISCUSSION

This thesis has aimed to investigate the molecular changes after homeostatic plasticity in hippocampal regions. Homeostatic plasticity has important roles in maintaining stable neuronal network functions and impairments can lead to neurological disorders (Ramocki and Zoghbi (2008)). Since the discovery of the mechanism in 1998 (Turrigiano et al. (1998)), homeostatic plasticity has received a lot of experimental attention. Although there has been a lot of progress in our understanding of homeostatic plasticity, in terms of its physiological and molecular mechanisms, it is still far from complete (Turrigiano (2008)). Moreover, the majority of the work until now was done in cell cultures. Although cell cultures are useful due to their technical ease of use for experiments, the neurones are in a very different environment from the natural environment in the intact brain which raises concerns over applicability of the in vitro results directly to in vivo settings.

Strategy for investigating gene expression changes during homeostatic plasticity in vivo

To investigate the gene expression changes during homeostatic plasticity I have over-expressed Kir2.1 in hippocampal neurones in vivo. This was done by infection of neurones with AAV or lentivirus expressing Kir2.1. Previous experiments investigating in vivo homeostatic plasticity have used pharmacological manipulations (Echegoyen et al. (2007)) or visual manipulations such as eye lid sutures (Hengen et al. (2013)). Pharmacological manipulation involved infusion of TTX which blocks action potentials in all the neurones. Similarly, eye lid sutures also removes inputs to the visual cortex hence reducing all input dependent activity in visual cortex. The manipulation by Kir2.1 expression has advantages over other methods in investigating gene expression changes. The major advantage is the targeting of the manipulation to neurones identified by injection site and virus promoter. This is in contrast to TTX infusion or eyelid suturing, where input to many cell circuit components is potentially modified and it is therefore difficult to distinguish primary from secondary adaptive changes. A further advantage of the approach is that it enables gene expression to be analysed in a defined cell type. It has been shown from many studies that the gene expression profile is different between different cell types (Sugino et al. (2006), Siegert et al. (2012) and Heiman et al. (2008)). As different cell types in the sample will gen-
erate base line noise in the gene expression experiment, it would ideal to focus the analysis on a single cell type. Also the manipulation may not affect all the cells in the region especially in the lid sutures. If the sample includes unaffected neurones this would also give background noise in the genetic profiling experiment. I have used mCherry tagged Kir2.1 thus labelling Kir2.1 over expressed neurones which can be used to isolated pure population of manipulated cells of single cell type hence increasing the sensitivity of the experiment.

AAV mediated delivery of Kir2.1 results in high infectious rate without any adverse effects in DG. In addition, this was done in the YFP-H line (Feng et al. (2000)) which expresses YFP in DG granule cells. YFP and mCherry fluorescent cells were isolated in FACs sorter to obtain a pure population of granule cells cells which have undergone plasticity. The RNA content can be analysed to reveal genetic changes during the adaptation. By using this strategy baseline noise from different cell types and from cells that did not undergo homeostatic plasticity was removed. A control virus was injected into the opposite hemisphere of the brain to the Kir2.1 virus and the subsequent RNA samples were analysed with a paired design. This controls for potential gene expression changes during virus infection, FACs sorting and mouse to mouse difference in gene expression. Gene expression profiling experiments were done with RNAseq which allows unbiased analysis of all genes in the transcriptome. The results presented in chapter 5 of this thesis shows that the RNA samples from the sorting were of high quality and the RNAseq readout is displays all the expected cell marker gene levels. Thus this strategy gives a very powerful tool for investigating gene expression changes in vivo with high sensitivity.

In addition to the gene expression profiling experiments, this strategy also allows electrophysiological analysis of the homeostatic plasticity in vivo through slice electrophysiology experiments. mCherry fluorescence can be used to target the virus infected cells to measure electrophysiological properties. Due to the efficiency of the infection not every cell was infected in the injected region. This can be taken advantage of and the near by uninfected cells can be used as control cells. This reduces variability due to the difference in the inputs that the cell receives and increases statistical power by allowing paired analysis for the results.

By combining gene manipulation by virus injection, slice electrophysiology and FACs, this provides a powerful strategy for manipulation of neuronal activity in vivo and subsequent analysis of gene expression profiles and electrophysiological profiles at a high sensitivity. This also allows linking of physiological readout and the gene
expression changes which allows analysis of candidate genes which may be responsible for the physiological read out in question.

*Adaptation in CA1 pyramidal cells and in DG granule cells uses distinct mechanisms*

It has been shown that there exists various strategies a neurone can use for homeostatic adaptation (Turrigiano (2011)). Kim and Tsien (2008) showed that, when CA3 pyramidal activity was suppressed by TTX infusion, there are different mechanisms of adaptation in synapses projecting to different regions, namely CA3 to dentate or CA3 to CA1 synapses respond by increasing the synaptic strength but CA3 to CA3 synapses respond by decreasing the synaptic weight. This suggests potential differences in the adaptive mechanisms between different synaptic pathways in the hippocampus.

The results in chapter 4 of this thesis suggests that there is cell type specificity in the mechanism of plasticity in response to suppression of activity. The results suggests that in response to Kir2.1 over expression, DG granule cells do not change their properties in the synapses. However there are evidences that there may a specific change in the input output ration at a very specific membrane potential (near the threshold of action potential). In contrast, the CA1 pyramidal cells showed an increase in the frequency of the mEPSC as previously observed in the TTX infused adult CA1 in vivo and in Kir2.1 over expressing pyramidal cells in hippocampal cultures (Echegoyen et al. (2007), Burrone et al. (2002)).

The work presented here does not show homeostatic plasticity directly in a sense that there is a measurement of base line before the manipulation, measurement straight after manipulation showing reduction of activity and homeostatic recovery to the baseline after homeostatic plasticity. However Kir2.1 over-expression has been used as a strategy to cause homeostatic plasticity in other settings where the authors have directly shown homeostatic plasticity (Burrone et al. (2002)). Also the fact that the mechanism of adaptation is same in the previous studies looking at homeostatic plasticity argues that the adaptation seen here, at least in the CA1, is of homeostatic nature. More importantly, the infected cells show significant differences from the near-by uninfected cells predicted by over-expression of Kir2.1 suggesting that the manipulation is restricted to the virus infected cells and not the uninfected cells. The neuronal model was used to predict the decrease in excitability but the stimulation experiments show that the threshold for action potential firing is same between the uninfected and infected neurones, suggesting a recovery to the baseline state. Al-
though not shown directly, these observations argue for the fact that the adaptation of DG granule cells and CA1 pyramidal cells in response to Kir2.1 over-expression \textit{in vivo} is homeostatic.

To my knowledge, this is the first direct demonstration of different excitatory cell types using different strategies for homeostatic plasticity in response to the same manipulation. The factor that determines which mechanism a cell will use is unclear. This may be due to the molecular profile differences between the two cell types or connectivity differences in the neuronal circuit.

\textit{Molecular changes in neuronal plasticity}

RNA-seq was used to analyse the molecular profile of pure population of adapted DG granule cells. The RNA sequences from the pure granule cell population showed high levels of granule cell markers and low levels of cell type markers of astrocytes, micro glia, pyramidal cells, interneurones and new born granule cells indicating that the samples were indeed from a pure granule cell population. Moreover differential expression analysis between the sorted DG granule cells and previously published sequences of hippocampal RNA (Stilling et al. (2014)) shows enrichment of GO terms associated neuronal parts such as dendrites and synapses. These results confirms the validity of the strategy by demonstrating that sorting enriches granule cell markers and depletes other cell type markers. The analysis of ERCC spike in inserted with the sample before the amplification process reveals a liner relationship between the RNA concentration in the sample and the number of reads generated from the sequence run further confirming the validity of the strategy.

Differential expression analysis between the Kir2.1 over-expressing DG granule cells and mCherry expressing DG granule cells show 210 differentially expressed genes in response to Kir2.1 expression. In the differentially expressed gene list there were genes such as CAMK4 and NOS1 which have been previously shown to be involved in homeostatic plasticity (Ibata et al. (2008), Steinert et al. (2011)). The physiology results from the Kir2.1 expressing DG granule cell suggested that the adaptation to decreased excitability is achieved through changing the input output properties at a specific resting membrane potential. O’Leary et al. (2014) showed that in a neuronal model, it is possible to obtain different spike firing patterns just by changing the expression level of ion channels. This lead me to hypothesise that the DG granule cells achieves this very specific change in intrinsic excitability by changing the expression level or the activity of voltage sensitive ion channels. Changes in ion channels in-
volved in ion conductance will have an impact on the input output relationship and if the ion channel is voltage sensitive this will give its selectivity on the membrane voltage. The differentially expressed gene list contained 13 differentially expressed genes which belonged to transporter activity GO term. Although none of the genes were previously linked directly with transporter activity GO term. Although none of the genes were previously linked directly with transporter activity GO term. Although none of the genes were previously linked directly with transporter activity GO term. Although none of the genes were previously linked directly with homeostatic plasticity, many have been linked to calcium buffering which is suggested as a potential sensor for neuronal activity (LeMasson et al. (1993)). This list still needs to be verified with qPCRs and the functional implication of each gene needs to be investigated to tease apart the precise mechanism of this plasticity.

This dataset provides, in my knowledge, the first candidate gene list which is involved in adaptation to suppression of activity in vivo. The genes in this list can be used in further studies as targets for investigation of molecular mechanisms of neuronal plasticity.

6.1 Future Work

The contents of this thesis demonstrate a powerful workflow for investigating physiological and genetic changes after gene manipulation in vivo. I have demonstrated this by analysing the molecular changes after suppression of neuronal activity in DG granule cells in vivo. I have also begun investigating the changes after the same manipulation in CA1 pyramidal cells. This leads to an interesting observation that neuronal subtypes adapt through distinct physiological mechanisms in response to the same manipulation. This most likely involves changes in different set of genes. A next aim is to investigate the gene expression changes in CA1 pyramidal cells. On going work from the lab shows that YFP-H mouse line is inadequate for this experiment due to the sparse expression of YFP in the CA1 pyramidal cells. To overcome this problem I have begun using Tg(Sim1-cre)KJ21Gsat/Mmucd mouse line (GENSAT) which expresses cre in CA1 pyramidal cells combined with a newly developed AAV-FLEX-Kir2.1-mCherry virus. Pilot sorting reveals that the samples are enriched with CA1 pyramidal cell marker (NeuroD6) and depleted of granule cell marker (MCM6), astrocyte marker (GFAP) and interneuron marker (GAD2). This work has been carried out in collaboration with Dr. Christina McClure. The CA1 gene expression profiling experiments will provide a set of gene involved in post synaptic changes involved in the increased frequency of mEPSC which has been widely observed in different preparations (Echegoyen et al. (2007), Burrone et al. (2002)). Also the physiological dataset needs to be strengthened with closer investigation in to the threshold for action potential firing and the synaptic current experiment needs to be repeated with
TTX to measure the mEPSC directly.

Possible pre-synaptic involvement raises interesting questions also. Work done by Kim and Tsien (2008) suggests that there are also synapse specific mechanism of adaptation. CA1 receives two major inputs, CA3 inputs through the schaffer collateral pathway and entorhinal inputs from the perforant pathway. Work in this thesis shows that pyramidal cells increase mEPSC frequency in response to Kir2.1 expression. When schaffer collateral is electrically stimulated it gives higher response in the Kir2.1 expressing pyramidal cells. This suggests that there is an increase in synaptic connection from the schaffer collateral or higher release probability possibly by increased number of presynaptic vesicles (Burrone et al. (2002)). Both would result in higher evoked EPSC as seen in this thesis. However, experiments where perforant pathway is stimulated will give insights into whether there is a synapse specific adaptation in CA1 pyramidal cells. Previous work (Kim and Tsien (2008)) has shown that there is a synapse specific adaptation in CA3 pyramidal cells following chronic application of TTX. As Kir2.1 does not affect any presynaptic neurones, comparing schaffer collateral adaptation and perforant pathway adaptation will potentially give insight in cross talk between pre and post synaptic neurones for homeostatic adaptation.

In addition to changing the transcriptional levels of genes, there may be other ways of modifying the levels of proteins which have functional impact in the neurone (Vogel and Marcotte (2012)). Proteomic studies can give a more representative picture of endpoint of changes during homeostatic plasticity by highlighting proteins which may have altered independently to the transcriptional change or through a downstream mechanism. A potentially interesting experiment would be to combine this system and the recently developed TRAP (translating ribosome affinity purification) method (Heiman et al. (2008)). TRAP utilises mouse lines where GFP is tagged to the ribosomes of specific cell types and specifically purifies mRNA which is bound to GFP ribosomes. This allows the analysis of translating RNA giving information about the rate of translation. The same strategy can be performed in a mouse line with GFP tagged ribosomes in CA1 pyramidal cells or DG granule cells and the cells can be sorted in the same was as YFP-H mouse line. TRAP reactions can then be performed on the sorted cells revealing ‘translatome’ that is involved in homeostatic plasticity which may or may not be similar to the transcriptome.
6.2 FINAL CONCLUSION

I have presented here a workflow for changing neuronal activity in vivo and investigating the physiological and genetic changes at a high sensitivity. This can potentially be used for many different questions in neuroscience where the neurones with manipulation can be tagged with a fluorescent protein. I aimed to investigate the molecular changes involved in neuronal plasticity in vivo. I, in collaboration with other members in the lab, demonstrated that different cell types (DG granule cells and CA1 pyramidal cells) use distinct mechanisms to adapt to the same manipulation and generated a RNAseq dataset for DG granule cells showing potential gene candidates responsible for the change. The list can be used for hypothesis driven studies to investigate molecules involved in neuronal plasticity in response to decreased excitability.
APPENDIX

Cuffdiff script

#Ran with 4 2G nodes
./cuffdiff -o Outcuffdiff/ -p 4 Mus_musculus.GRCm38.74.gtf 1192N0001_sort_chr.bam,1192N0003_sort_chr.bam,1192N0005_sort_chr.bam,1192N0007_sort_chr.bam,1192N0002_sort_chr.bam,1192N0004_sort_chr.bam,1192N0006_sort_chr.bam,1192N0008_sort_chr.bam

DESeq script

> library("DESeq2", lib.loc="/Library/Frameworks/R.framework/Versions/3.1/Resources/library")
> deseq2.dds <- DESeqDataSetFromMatrix(countData = deseq.Counttable, colData = deseq.Design, design = ~ condition + mouse)
> colData(deseq2.dds)
DataFrame with 8 rows and 4 columns

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> colData(deseq2.dds)$condition <- relevel(colData(deseq2.dds)$condition, "mCherry")
> deseq2.ddsMF <- deseq2.dds
> design(deseq2.ddsMF) <-formula (~mouse + condition)
> colData(deseq2.ddsMF)$condition <- relevel(colData(deseq2.ddsMF)$condition,
"mCherry")
> deseq2.ddsMF <- DESeq(deseq2.ddsMF)
estimating size factors
estimating dispersions
gene-wise dispersion estimates
mean-dispersion relationship
final dispersion estimates
fitting model and testing
> deseq2.resMF <- results(deseq2.ddsMF)
> write.csv(as.data.frame(deseq2.resMF), file="deseq2paireddesign.csv")

edgeR script

> virus <- factor(c("Kir","Kir","Kir","mCherry","mCherry","mCherry","mCherry"))
> mice <- factor(c(1,2,3,4,1,2,3,4))
> edr.y <- DGEList (deseq.Counttable)
> edr.y
An object of class "DGEList"

$counts

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<td>1</td>
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<td>mCherry3</td>
<td>11832115</td>
<td>1</td>
</tr>
</tbody>
</table>
\begin{verbatim}
> edr.y$samples$lib.size <- colSums(edr.y$counts)
> edr.y <- calcNormFactors(edr.y)
> edr.y$samples

    group lib.size    norm.factors
  Kir1  1 21129814 0.9775451
  Kir2  1 20681100 0.9582920
  Kir3  1 22126598 0.9270844
  Kir4  1 18758974 1.0368512
 mCherry1 1 23619644 0.9391756
 mCherry2 1 17426594 1.0015481
 mCherry3 1 11832115 1.0944581
 mCherry4 1 20745011 1.0787276

> data.frame(Sample=colnames(edr.y), mice, virus)

Sample mice virus
 1 Kir1 1 Kir
 2 Kir2 2 Kir
 3 Kir3 3 Kir
 4 Kir4 4 Kir
 5 mCherry1 1 mCherry
 6 mCherry2 2 mCherry
 7 mCherry3 3 mCherry
 8 mCherry4 4 mCherry

> edr.design <- model.matrix(~mice+virus)
> edr.y <- estimateGLMCommonDisp(edr.y, edr.design, verbose=TRUE)
  Disp = 0.6154 , BCV = 0.7845
> edr.y <- estimateGLMTrendedDisp(edr.y, edr.design)
> edr.y <- estimateGLMTagwiseDisp(edr.y, edr.design)
> fit <- glmFit(edr.y, edr.design)
> lrt <- glmLRT(fit)
> edr.outtab <- cbind(lrt$table, FDR=p.adjust(lrt$table$PValue, method="BH"))
> write.csv(as.data.frame(edr.outtab), file="edr.csv")
\end{verbatim}
| ENSMUSG00000041695 | Kcnj2 | 17229.65821 | 3.882350084 | 2.65E-84 | 4.32E-80 |
| ENSMUSG00000040600 | Eps8l3 | 73.95094827 | -6.581713596 | 2.93E-10 | 2.38E-06 |
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| ENSMUSG00000089886 | Gm16184 | 188.3221359 | 4.974996543 | 2.30E-09 | 9.35E-06 |
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Müller, M., Genç, O., and Davis, G. (2013). RIM-Binding Protein Links Synaptic Homeostasis to the Stabilization and Replenishment of High Release Probability


