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Elucidating the Reversibility of Ataxia

by

Daumantė Šuminaitė

THE UNIVERSITY of EDINBURGH

A thesis submitted for the degree of Doctor of Philosophy

2017
Declaration

I declare that this thesis was solely composed by myself and the work was carried out by me, unless otherwise acknowledged. None of this work has been submitted for any other degree qualification.

Daumantė Šuminaitė
April 2017
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Abstract

Heterozygous and recently identified homozygous mutations in the SPTBN2 gene, encoding β-III spectrin, are implicated in spinocerebellar ataxia type 5 (SCA5) and spectrin-associated autosomal recessive cerebellar ataxia type 1 (SPARCA1), respectively. Our mouse model, lacking β-III spectrin (KO), mimics the progressive human phenotype displaying motor deficiencies as well as reduced Purkinje cell firing frequency followed by dendritic tree degeneration and cell death. The aims of this study were to evaluate progression of Purkinje cell degeneration following loss of β-III spectrin function and determine whether the reintroduction of C-terminus (C-trm) of β-III spectrin to the cerebellum is enough to halt, alleviate or reverse the disease phenotype. Additionally, this study investigated whether the abnormal electrophysiological and morphological phenotypes of Purkinje cells from KO mice are re-capitulated in a primary cerebellar culture and if so, whether they could be rescued by modulating calcium signaling.

Morphological and histological analyses revealed that Purkinje cell degeneration is not uniform throughout the cerebellum of KO mice with Purkinje cells from posterior cerebellar regions possessing significantly smaller dendritic trees when compared to anterior cerebellum (p=0.0003, N=4-6, n=11-29). Similarly, significant reduction in Purkinje cell density was observed in posterior, not anterior regions of KO mice when compared to WT animals (p=0.014, N=3) and reduced tonic firing is most significant in Purkinje cells from the posterior cerebellum compared to WT mice (p=0.0328, N=3-6, n=11-29), with posterior KO PCs appearing to have elevated input resistance.

Two-week expression of C-trm β-III spectrin in 3-month old KO animals signifi-
cantly reduced Purkinje cell input resistance when compared to non-transduced cells (p=0.0139, N=4-5, n=15), but no effect was seen 9 months after viral injection. In contrast, a difference in cell surface area was no longer detected between WT and KO animals at 12 months of age following 9-months of viral expression. Nevertheless, using the elevated beam test motor deterioration was still observed 5 months after surgery (p=0.0023, N=4). In contrast, earlier stereotaxic injections at 6-weeks of age had a positive effect on mice motor performance with no deterioration in performance detected 5 months after the surgery. Latency to stay on the rotarod at 3 rpm was also significantly extended 6 months after stereotaxic injections at 6-weeks of age with slower motor deterioration (p=0.0348, N=6).

In primary cerebellar cultures, Purkinje cells from KO animals exhibit an abnormal morphology with significantly more dendritic branches (p<0.0001, N=4-7, n=35-69) and a larger total dendritic length (p=0.0079). Chronic application of 2 µM mibebradil, a T-type calcium channel blocker, was observed to reduce total dendritic length and branching in KO animal cultures bringing these morphological measurements closer to WT Purkinje cell levels. Finally although after 14 days in vitro 40% of Purkinje cells were found to be spontaneously firing, no significant difference in firing frequency (p=0.9434) or input resistance (p=0.8434, N=4, n=6-10) was detectable between WT and KO cultures.

In summary, Purkinje cells in posterior cerebellar regions of KO mice were found to be more susceptible to dendritic degeneration and cellular death than cells in the anterior cerebellum. Expression of C-trm β-III spectrin at 3 months of age had an immediate effect on cell input resistance and a modest effect on Purkinje cell morphology but no effect on motor decline. Viral injections at 6-weeks of age, however, significantly slowed motor decline. Although an abnormal KO cell morphology could be successfully recapitulated in primary cell culture, it was not possible to discern any differences in electrophysiological properties. Nevertheless, the abnormal cell morphology was successfully modified in vitro by manipulating calcium signaling via T-type calcium channels.
Lay Summary

Alterations to a protein, \( \beta \)-III spectrin, have been found to give rise to disorders of the brain that result in a progressive loss of balance and uncoordinated movement. The cerebellum is the part of the brain that is important for these functions and \( \beta \)-III spectrin function has been shown to be critical for the normal function of the cerebellum. Our mouse model, in which animals lack \( \beta \)-III spectrin protein, also exhibits progressive motor decline and Purkinje cell death re-capitulating the clinical disease. This animal model has enabled the time line of disease to be studied and has revealed that Purkinje cells, the key nerve cells of the cerebellum, when lacking \( \beta \)-III spectrin function have a different shape, do not function properly, then they begin to degenerate and eventually they die. The aim of this study was to further understand the disease time line and to identify therapeutic approaches that might alleviate the condition and improve patient symptoms.

The study revealed that Purkinje cell death is not uniform throughout the cerebellum but occurs first in the bottom (posterior) regions, suggesting that therapeutic approaches should target specific regions rather than the whole cerebellum. It was also observed that reintroduction of a part of the \( \beta \)-III spectrin protein was sufficient to reduce the motor in-coordination in mice but only when introduced early in adulthood, suggesting a possible therapeutic window.

When cell death mechanisms were analysed in a cell culture model, it was shown that our culture model successfully mimics some of the Purkinje cell shape abnormalities. It was also found that a drug, that alters calcium levels within cells, was able to alter the shape of Purkinje cells lacking \( \beta \)-III spectrin, making them look more like normal
cells. These findings illustrate that this cell culture system can be used for various drug manipulations in order to discover successful therapeutic strategies to treat disorders resulting in decline of motor coordination.

In summary, this study showed that certain cerebellar regions are more prone to cell death than others, suggesting targeted therapeutic approaches are appropriate. It was also found that reintroduction of even a part of β-III spectrin protein early in life reduces motor decline suggesting that therapeutic interventions in patients should take place as early as possible. Additionally, the study found that manipulating calcium levels in Purkinje cells lacking β-III spectrin was able to restore cell shape towards that of healthy Purkinje cells.
Acknowledgments

Although this thesis carries a name of only one author, many people have helped me over the course of my PhD studies.

First of all, I would like to extend my sincerest gratitude to my supervisor and my mentor, Dr Mandy Jackson, for her incredible patience, support and guidance through these years.

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I would also like to acknowledge Dr Paul Skehel, whose door was always open and who was always ready to share his encyclopedia-like molecular biology knowledge. My special thanks are also extended to Dr Christina McClure and Dr Matthew Nolan for providing me with reagents and equipment needed for viral particle production.

Additionally, I would like to thank my partner, Gintas Sasnauskas, who taught me MATLAB and encouraged me along the way.

And finally, I would like to thank my family, who always believed in me and supported all of my decisions. Ačiū Jums.
Abbreviations

AAV Adeno-associated virus
ACSF artificial cerebrospinal fluid
ADCA Autosomal dominant cerebellar ataxias
AFG3L2 AFG3 like matrix AAA peptidase subunit 2
ALS Amyotrophic lateral sclerosis
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APS ammonium persulphate
b Base
BK channel Voltage- and Ca\(^{2+}\) activated K\(^{+}\) channel
BS Blocking solution
BSA Bovine serum albumin
CAG Glutamine
CaMKII Ca\(^{2+}\)/calmodulin-dependent protein kinase II
CMV Cytomegalovirus
CNS Central nervous system
C-trm Carboxy-terminus
CV Coefficient of variation
Cy Cyanine
CβAG CMV early enhancer/chicken β actin promoter
Da Dalton
DIV Days *in vitro*
DMEM Dulbecco’s modified eagle medium
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DsRed</td>
<td><em>Discosoma sp.</em> red fluorescent protein</td>
</tr>
<tr>
<td>EA2</td>
<td>Episodic ataxia type 2</td>
</tr>
<tr>
<td>EAAT4</td>
<td>Excitatory amino acid transporter 4</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory post-synaptic currents</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>eSYN</td>
<td>Enhanced synapsin promoter</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FFA</td>
<td>Flufenamic acid</td>
</tr>
<tr>
<td>FGF14</td>
<td>Fibroblast growth factor 14</td>
</tr>
<tr>
<td>FL</td>
<td>Full length</td>
</tr>
<tr>
<td>FL WO</td>
<td>Full length wrong orientation</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-Aminobutyric acid</td>
</tr>
<tr>
<td>GALC</td>
<td>Galactocerebrosidase enzyme</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIRK2</td>
<td>G-protein-regulated inward-rectifier potassium channel 2</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>GluR(\delta)2</td>
<td>(\delta)2 glutamate receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HEBs</td>
<td>HEPES-buffered saline</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cell line, derived from Henrietta Lacks’ cervical cancer cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
</tr>
<tr>
<td>HHS</td>
<td>Hank’s/Hepes solution</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Horse serum</td>
</tr>
<tr>
<td>I/O</td>
<td>Input/output</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>IMPPase</td>
<td>inosinic acid pyrophosphorylase</td>
</tr>
<tr>
<td>IR</td>
<td>Input resistance</td>
</tr>
<tr>
<td>IRES eGFP</td>
<td>Internal ribosome entry site enhanced green fluorescent protein</td>
</tr>
<tr>
<td>iRNA</td>
<td>interference RNA</td>
</tr>
<tr>
<td>ISI</td>
<td>interspike interval</td>
</tr>
<tr>
<td>ITPR</td>
<td>inositol 1,4,5- trisphosphate receptor</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
</tr>
<tr>
<td>k</td>
<td>Kilo</td>
</tr>
<tr>
<td>KCNC3</td>
<td>potassium voltage-gated channel subfamily C Member 3</td>
</tr>
<tr>
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<td>Knock-down</td>
</tr>
<tr>
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<td>Knock-in</td>
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<tr>
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<td>CaMKII blocker</td>
</tr>
<tr>
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<td>Knock-out</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby canine kidney</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium eagle</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
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<td>mibefradil</td>
<td>T-type Ca^{2+} channel blocker</td>
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<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
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<tr>
<td>ML</td>
<td>months later</td>
</tr>
<tr>
<td>mo</td>
<td>Month</td>
</tr>
<tr>
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</tr>
<tr>
<td>NBQX</td>
<td>AMPA receptor blocker</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
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<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NPC</td>
<td>Niemann-Pick disease type C</td>
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<tr>
<td>NRK</td>
<td><em>Rattus norvegicus</em> kidney cells</td>
</tr>
<tr>
<td>N-trm</td>
<td>N-terminus</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cell</td>
</tr>
<tr>
<td>pcd</td>
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<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>Protein kinase C</td>
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<td>Phospholipase C</td>
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<td>Phospholipase C&lt;sub&gt;β4&lt;/sub&gt;</td>
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<tr>
<td>polyQ</td>
<td>Poly-glutamine tract</td>
</tr>
<tr>
<td>PTX</td>
<td>Picrotoxin (a noncompetitive antagonist of GABA&lt;sub&gt;A&lt;/sub&gt; receptors)</td>
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<tr>
<td>rAAV</td>
<td>Recombinant adeno-associated virus</td>
</tr>
<tr>
<td>Rac</td>
<td>Subfamily of Rho GTPases</td>
</tr>
<tr>
<td>RBM17</td>
<td>RNA binding motif protein 17</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SCA</td>
<td>Spinocerebellar ataxia</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>sg</td>
<td>Staggerer</td>
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<tr>
<td>SH3</td>
<td>Src homology-3 domain</td>
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<td>SPARCA1</td>
<td>Spectrin-associated autosomal recessive cerebellar ataxia type I</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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<td>TAR DNA-binding protein 43</td>
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<td>Untransfected</td>
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<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>wk</td>
<td>Week</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Chapter 1

Introduction

1.1 Cerebellum

1.1.1 Introduction to cerebellum

The cerebellum, or "little brain" is a brain structure overlying posterior parts of pons and medulla in humans (Colin et al., 2001). It accounts for 10% of total brain volume (Llinás et al., 2004), but harbours more than half of the total brain neurons (Herculano-Houzel, 2009). In contrast, relatively small numbers of glial cells are discovered in the cerebellum, with neurons outnumbering them 25 to 1 (Andersen et al., 1992). Such big numbers of neurons are tightly packed by folding the cerebellar surface giving it its distinct appearance with the grooves and fissures separating 260 different parallel folds, or cerebellar folia as seen in Fig. 1.1 (Colin et al., 2001; Kandel et al., 2013). The cerebellum is made of two distinct elements - cerebellar cortex and cerebellar nuclei - fastigial, interposed and dentate (Llinás et al., 2004; Kandel et al., 2013). The cerebellar cortex can be divided into 3 layers: molecular, Purkinje cell and granular (Fig. 1.2).
1.1.2 Historic functions of the cerebellum

Before discussing currently known cerebellar anatomy and function, a brief history of the cerebellar research will be presented. The interest in the cerebellum and its anatomy started in the middle of the XVI century (Glickstein et al., 2009) and by the XVIII century there were accurate depictions of cerebellar anatomy, however, its functions remained unknown. One of the earlier experimental studies on cerebellum by Rolando (1809) recognised that cerebellar lesions resulted in motor symptoms. He proposed that cerebellum is responsible for initiating movement (Rolando, 1809). Experiments by Pierre Fluorens in the XIX century observed the absence of paralysis following the cerebellar lesion, therefore he suggested cerebellar function to be movement coordination rather than initiation (Fluorens, 1824). Fluorens also reported that chickens are able to recover from partial, but not full cerebellar removal as reviewed by Ito (2002). These experiments suggested cerebellar learning abilities.

Further studies of Luciani have identified that the cerebellar lesions have long lasting effects such as atonia (loss of muscle tone) and intention tremor (Luciani, 1891). In the rare cases where cerebellar agenesis have been reported, it was observed that patients were able to walk and had motor skills. The patients, however, were only reported to start walking at later ages (3 and 7 years of age) (Anton and Zingerle, 1914; Tennstedt, 1965), suggesting that the cerebellum is essential for normal motor development.
Cerebellar abnormalities

Cerebellar abnormalities manifest in disturbance to normal movement, originally observed by Rolando (1809) who noted that cerebellar lesions impaired the movement. Descriptions from Babinski (1899) and Gordon Holmes (1917), however, showed the hindrance in normal movement sequence, rather than complete abolishment (Kandel et al., 2013). Cerebellar disorders have detrimental consequences for performing the simplest sequences of movements (Llinás et al., 2004), therefore cerebellum plays an important role in motor coordination (Llinás et al., 2004) and is sometimes described as playing the assistive role in movement (Manto, 2005).

1.1.3 Purkinje cells

Although the cerebellum has undergone elaborate evolution (from 20 folia in pigeons to 260 in humans (Colin et al., 2001)), its primary neuronal structure has remained the same and is present in all vertebrates (Llinás et al., 2004). The principal neurons are Purkinje cells which were first observed in 1837 by Jan Evangelista Purkinje (Purkinje, 1838) and human cerebellum is estimated to contain 15 million of them (Colin et al., 2001). Their somas (30 µm in diameter) are tightly packed in a Purkinje cell monolayer spanning the whole cerebellar cortex as seen in Fig. 1.2 (Colin et al., 2001). Their elaborate dendritic trees, described in detail by Camillo Golgi (Golgi and Fischer, 1894) and spanning 300 µm in diameter, project through the molecular layer (Apps and Garwicz, 2005). Purkinje cell axons are the sole output of the cerebellum (Ramón y Cajal, 1904) and send GABAergic projections (Ito and Yoshida, 1964) to vestibular and deep cerebellar nuclei (Colin et al., 2001). Some Purkinje cell collaterals form synapses with and inhibit other Purkinje cells (Bernard and Axelrad, 1993).

1.1.4 Cerebellar inputs

Climbing fibres

Although the Purkinje cells supply sole cerebellar output, their activity is heavily influenced by the excitatory and inhibitory inputs. The excitatory cerebellar input
comes in via two pathways - climbing fibres and mossy fibres. Climbing fibres arise from inferior olive nucleus in brainstem, with inferior nucleus receiving inputs from spinal cord, motor cortex, brainstem and cerebellar nuclei (Llinás et al., 2004). Climbing fibres wrap around the planar Purkinje cell dendritic tree in the molecular layer as seen in Fig. 1.2 (Apps and Garwicz, 2005) and are considered the most powerful excitatory synapse in the brain (Colin et al., 2001). Although during development multiple climbing fibres innervate one Purkinje cell (Crepel, 1982) in the mature cerebellum only one climbing fibre is in contact with one Purkinje cell (Apps and Garwicz, 2005). However, each cell in the inferior olive gives rise to approximately 10 climbing fibres. Each climbing fibre innervates a cluster of Purkinje cells in embryo, which in adult becomes stripes of Purkinje cells, seen in coronal slices, when stained for phospholipase C (PLC) or zebrin (Apps and Hawkes, 2009). Climbing fibre input underlies complex spikes observed in Purkinje cells (Eccles et al., 1966b; Thach W. T., 1967). Lesions and abnormalities in inferior olive and climbing fibre input results in disorders resembling cerebellar abnormalities (Section 1.3) (Wilson and Magoun, 1945; Apps and Garwicz, 2005).

Mossy fibres

Mossy fibres, originating in the central nervous system (CNS) (Llinás et al., 2004) enter the cerebellum through the cerebellar peduncles making excitatory synapses with Golgi cells and granule cells in the granular layer (below the Purkinje cell layer, Fig. 1.2). The granular layer is formed by densely packed granule cells which project to the molecular layer and form parallel fibres, which run perpendicularly to the dendritic trees of Purkinje cells (Llinás et al., 2004) and synapse onto Purkinje cells as seen in Fig. 1.3 (Apps and Garwicz, 2005). Each Purkinje cell is estimated to make contacts with approximately 200,000 granule cells. Indirect mossy fibre innervation of Purkinje cells is responsible for simple Purkinje cell spike firing (Llinás et al., 2004). Mossy and climbing fibres also extend collaterals into deep cerebellar nuclei modifying Purkinje cell output (Shinoda et al., 1992; Llinás et al., 2004; Apps and Garwicz, 2005).
Local inhibitory inputs

While mossy and parallel fibre inputs onto Purkinje cells are excitatory, inhibitory neurons in local cerebellar circuitry are also able to modify Purkinje cell output (Purves et al., 2001). Golgi cells, the cell bodies of which lie in the granular layer (Eccles et al., 1967), have extensive dendritic trees which not only reside in the granular layer, but also the molecular cell layer (Eccles et al., 1967). Golgi cells receive glutamatergic inputs from the parallel fibres in the molecular cell layer (Dieudonne, 1998) and mossy fibres in the granular (Kanichay and Silver, 2008). In turn, Golgi cells project their dendrites onto granule cells, where they inhibit granule-mossy cell synapses (Eccles et al., 1967) via GABA_\text{A} receptors (Brickley et al., 1996) with fast and sustained inhibition (Crowley et al., 2009). This feedback inhibition is expected to reduce mossy fibre input onto Purkinje cells (Marr, 1969; Gabbiani et al., 1994) and mice with Golgi cells ablated
Figuré 1.3: Neuronal circuity of the cerebellum. Climbing fibres originating from the inferior olive nucleus project to the molecular layer where they wrap around and form excitatory synapses with Purkinje cells. Mossy fibres, projecting from brain stem and spinal cord through cerebellar peduncles, form excitatory synapses with granule cells in granular layer, which in turn project to the molecular layer forming co-lateral parallel fibres. Parallel fibres make connections with multiple Purkinje cells. Image adapted from Apps and Garwicz (2005).

have severe defects in motor function (Watanabe et al., 1998).

In the molecular layer, two types of inhibitory interneurons are distinguished (Eccles et al., 1966b; Vincent and Marty, 1996; Pouzat and Hestrin, 1997). Stellate (Midtgaard, 1992) and basket cells are both activated through glutamatergic synapses with parallel fibres (Eccles et al., 1966a) and form GABAergic synapses with Purkinje cell dendrites and bodies, respectively (Purves et al., 2001). Stellate and basket cells are therefore
Chapter 1 General introduction

providing feed-forward inhibition (modifying output), while Golgi cells provide feedback inhibition (modifying input) (Purves et al., 2001).

1.2 Spectrin

1.2.1 Spectrin subunits

This thesis concentrates on β-III spectrin, which is encoded by the SPTBN2 gene and was identified by Ohara et al. (1998). Spectrins are cytoskeletal proteins that were first discovered in erythrocytes and have been proposed to play a key role in maintaining cell shape throughout erythrocyte’s 120-day long life cycle, encompassing constant deformation, some of which requires a squeeze of a 8 µm diameter erythrocyte through 2 µm diameter capillaries (Marchesi and Steers Jr., 1968; Byers and Branton, 1985; Goodman et al., 1995; Machnicka et al., 2014). Spectrins form a supportive 2D latticework underneath the erythrocyte membrane (Byers and Branton, 1985; Xu et al., 2013) and erythroid spectrin loss results in shape instability causing anemia in humans (Tse and Lux, 1999; Gallagher, 2004). Soon after, spectrins in non-erythroid tissue were discovered in chicken cardiac myocytes (Goodman et al., 1981) and the spectrin cytoskeleton has been proposed to play a crucial role during neuronal migration and development (Hülsmeier et al., 2007). To date, seven genes encoding spectrins have been found in vertebrates with two alpha (α-I and α-II) and five β spectrins (β-I - β-V). α-I is primarily expressed in erythrocytes, while α-II is found in other tissues, with strong expression in the brain (Berghs et al., 2000). β-I is found in erythrocytes (Burridge et al., 1982), β-II is in the brain (Berghs et al., 2000), β-III expression has been shown in kidneys and the brain (Ohara et al., 1998), β-IV (Berghs et al., 2000) is found in pancreatic islets and the brain, while β-V is expressed in retina and gastric epithelial cells at high levels and at low level in other tissues (Stabach and Morrow, 2000). Differential splicing of spectrins also results in wide distribution with various isoforms expressed in different tissues (Berghs et al., 2000).
1.2.2 Mature spectrin protein

To form a mature spectrin protein, the N-terminus of α spectrin binds to the C-terminus of β spectrin and two such heterodimers bind head to head to form a tetramer (Tse et al., 1990). α and β spectrins contain multiple spectrin repeats (20 and 17 respectively), each comprised of 106 amino acids (Speicher and Marchesi, 1984). The interaction between the repeats in α and β subunits underlies the helix formation, which in turn is thought to play a key role in spectrin molecule stability (Yan et al., 1993). In the cerebellum, α-II and β-III spectrin form the mature protein of which schematic diagram is shown in Fig. 1.4. β-III expression was identified to be strongest in the brain as well as kidneys (Ohara et al., 1998). Within the brain, although cerebral cortex showed some β-III expression, cerebellar Purkinje cells had the highest levels of spectrin expression, with its localization seen in cell body and dendritic tree (Ohara et al., 1998).

![Diagram of α-II and β-III spectrin heterodimers binding head to head to form a mature spectrin protein with dimerisation and tetramerisation domains marked. ANK indicates ankyrin binding domain. Adapted from Lise et al. (2012).](image)

**Figure 1.4:** Diagram of α-II and β-III spectrin heterodimers binding head to head to form a mature spectrin protein with dimerisation and tetramerisation domains marked. ANK indicates ankyrin binding domain. Adapted from Lise et al. (2012).

1.2.3 Spectrin binding partners

Spectrins have multiple binding partners allowing them to cross-link actin and other proteins as reviewed in Perkins et al. (2016a). For example, α spectrin in repeat 9 contains a Src homology-3 (SH3) domain (Robertsson et al., 2005), which is implicated in protein-protein interactions (Pawson and Gish, 1992) and plays a role in Rac activation resulting in cell adhesion (Bialkowska et al., 2005).

The carboxy tail of β spectrins contains a pleckstrin homology (PH) domain (Macias et al., 1994). PH domain is a similar sequence of 100-120 amino acids found in proteins...
involved in cytoskeleton formation and cell signaling (Haslam et al., 1993). A PH domain is also suggested to be involved in PtdIns(4,5)P (PIP$_2$) binding (Harlan et al., 1994) and spectrin’s binding to the lipids of plasma membrane (Williams et al., 2004), however other PH functions still remain to be elucidated. The N-terminus of β spectrins also contain actin binding domains (Byers and Branton, 1985; Karinch et al., 1990). β-III spectrin has been found to co-localise with Golgi compartment markers (Stankevich et al., 1998) in Madin Darby canine kidney (MDCK), HeLa, and Rattus norvegicus kidney (NRK) cells suggesting tight association with Golgi. Association with other membrane organelles such as transport vesicles has also been observed (Stankevich et al., 1998; Lorenzo et al., 2010).

**Excitatory amino acid transporter 4**

Excitatory amino acid transporter 4 (EAAT4) has been demonstrated to localise to the CNS with high levels of expression in the cerebellar Purkinje cells (Furuta et al., 1997). The somatodendritic localisation of EAAT4 (Furuta et al., 1997) has been found to be the densest at the Purkinje cell dendritic spine membrane facing astrocytes (Dehnes et al., 1998), where it plays a key role in glutamate uptake at Purkinje cells synapses with climbing and parallel fibres (Huang et al., 2004; Takayasu et al., 2005; Power and Empson, 2014). β-III spectrin has been shown to co-precipitate with and stabilise EAAT4 at the cell membrane preventing its internalisation (Jackson et al., 2001). EAAT4 lacking the last 11 amino acids has been demonstrated not to be able to interact with β-III and is, therefore, not trafficked to the cell membrane (Jackson et al., 2001; Perkins et al., 2016b).

**Dynactin**

The microtubule-based motor protein dynein has been demonstrated to play a role in neuronal retrograde transport, cytokinesis and endoplasmic reticulum-to-Golgi vesicular trafficking (Holleran et al., 1998). Dynactin, an accessory protein, binds dynein as well as microtubules (Karki and Holzbaur, 1995; Waterman-Storer et al., 1995) and
is involved in dynein mediated transport (Waterman-Storer et al., 1997). β-III spectrin has been shown to co-precipitate with Arp1 subunit of dynactin and provide an interaction between membrane vesicles and dynactin (Holleran et al., 2001).

**Ankyrin**

In 1979 a study by Bennett and Stenbuck identified a novel spectrin binding partner - ankyrin, which localises to the erythrocyte membrane surface. The ankyrin binding domain is within the 15th spectrin repeat of β spectrin (Kennedy et al., 1991) and although being folded the same way as other repeats, the 15th spectrin repeat exposes distinct residues on its surface facilitating spectrin’s interaction with ankyrin (Bennett and Healy, 2009). Ankyrin has also been shown to bind to anion channel band 3 (Bennett and Stenbuck, 1979), being a bridge between a membrane spanning protein and the cell cytoskeleton (Davis and Bennett, 1984). In addition, ankyrin is also able to bind microtubules (Bennett and Davis, 1981). It is for these reasons that defects in both ankyrin and spectrin binding cause fragile erythrocytes to have significantly shorter lifespan and reduced spectrin levels (White et al., 1990). The same authors later identified an isoform of ankyrin expressed in the brain, ankyrin B, and it was also found to possess anion channel as well as microtubule binding domains (Davis and Bennett, 1984). Subsequently, ankyrins G and R have been demonstrated to bind the Na, K ATPase (Nelson and Veshnock, 1987; Zhang et al., 1998) and voltage-gated sodium channels (Srinivasan et al., 1988).

An association between ankyrin G and β-IV spectrin at the axon initial segment has been demonstrated in Purkinje cells (Jenkins and Bennett, 2001). Together, ankyrin G and β-IV spectrin have been found to stabilise each other as well as mutually direct voltage-gated sodium channels to axon initial segments and nodes of Ranvier (Komada and Soriano, 2002).

Similarly, in the dendritic tree of Purkinje cells β-III spectrin has been found to recruit ankyrin R to the plasma membrane and in the β-III spectrin spectrin knock-out (KO) mouse model ankyrin R levels have been shown to be reduced throughout the
molecular layer (Clarkson et al., 2014). Furthermore, sodium currents in dissociated
hippocampal cultures were reported to increase when ankyrin R and β-III spectrin were
co-expressed, indicating a requirement for a β-III spectrin/ankyrin R complex in the
stabilisation of voltage-gated sodium channels (Clarkson et al., 2014).

1.2.4 Animal studies of β-III spectrin

Multiple animal studies have highlighted that similar to erythroid spectrin, β-III spec-
trin is critical for cell shape, with aberrant cell morphology and placement observed in
KO and knock-down (KD) animals. Electrophysiological studies suggest, that animals
lacking β-III spectrin have exaggerated miniature postsynaptic potentials as well as
dysregulation in glutamate receptor expression.

Caenorhabditis elegans

As spectrin homologues are found in various animal species, one α and two β spectrin
subunits (G and H) have been identified in Caenorhabditis elegans (C. elegans). Studies
carried out confirm that spectrin is critical for normal muscle and neuron function. A
study by Moorthy et al. (2000) demonstrated that β-G spectrin was localized to the
plasma membrane at cell-cell contact sites starting at embryonic stages and lasting
into adulthood with strongest staining observed in the nervous system (Moorthy et
al., 2000). Introduction of interference RNA (iRNA) leading to β-G depletion, resulted
in abnormal actin filament distribution in muscle tissue. Furthermore, in the nervous
system, neuronal cells were observed to be enlarged, sometimes displaced and showed
signs of vacuolisation (Moorthy et al., 2000).

Furthermore, mutation within the β-G gene, unc-70, results in phenotypically β-G
null C. elegans and abnormal placement of GABAergic neurons in the ventral cord
(Hammarlund et al., 2000). In addition, the axonal and commissural outgrowths in
ventral and dorsal cords were observed to be defasciculated and with abnormal mor-
phologies. Some neurons were found to terminate prematurely and not reach their
normal targets (Hammarlund et al., 2000). A further study by the same authors re-
vealed that some axon comissures from dorsal cord were detached indicating axonal breaking as well as aberrant branching (Hammarlund et al., 2007). These findings indicate that β-G spectrin is crucial for protecting neurons from breaking which can happen due to movement induced strain during neuronal development.

**Drosophila melanogaster**

In *Drosophila*, one α and two β spectrins (β and β-H) have been identified (Dubreuil et al., 2000). Mutations introduced into β spectrin showed that Na, K ATPase localisation was severely affected which was a result of β spectrin loss-of-function (Dubreuil et al., 2000). As global α and β spectrin KO larvae died during embryogenesis (Lee et al., 1993), iRNA was used to diminish spectrin expression levels at the post-synaptic membrane of the neuromuscular junction (Pielage et al., 2006). The authors observed that in α or β KD, the number of synaptic boutons at the neuromuscular junctions were significantly reduced and the active zones at the synapses were significantly larger (Pielage et al., 2006). Furthermore, in the absence of postsynaptic β spectrin, the size of glutamate receptor clusters was significantly increased. Electrophysiological recordings from these junctions demonstrated that miniature excitatory postsynaptic potential was also significantly increased in β spectrin KDs. In line with this, neuronal response to single action potential was also dramatically increased. The loss of recruited ankyrin to the membrane was also observed following β spectrin KD (Pielage et al., 2006).

Conversely, a study by Pielage et al. (2005) looked at the presynaptic membrane of *Drosophila* neuromuscular junction. It was observed that β spectrin loss from presynaptic terminal led to axonal retraction and synapse elimination. In the case of synapse retraction, no spontaneous or evoked transmitter release was observed with no changes to passive membrane properties. The microtubule skeleton was also observed to be severely disorganized. In both sensory and motor neurons, downregulation of spectrin appeared to lead to blocked microtubule transport.

In the *Drosophila* model of SCA5, neurodegeneration was also reported (Lorenzo et al., 2010). When full-length (FL) β-III spectrin carrying American or German mu-
tations was introduced into *Drosophila*, progressive neurodegeneration was observed in retinal neurons. As previously observed by Pielage et al. (2006), the number of synaptic boutons was also reduced in larvae with β-III spectrin mutations. The larvae also had a ‘tail-flip’ phenotype which has been associated with impaired axonal transport (Kurd and Saxton, 1996) and is indicative of motor impairment. The synaptic vesicles were observed to travel much shorter distances and reverse their travel direction frequently illustrating synaptic transport disruption (Lorenzo et al., 2010).

### 1.3 Spinocerebellar ataxias

#### 1.3.1 Introduction to SCAs

Mutations in gene encoding β-III spectrin results in spinocerebellar ataxia type 5 (SCA5) (Ranum et al., 1994; Stevanin et al., 1999; Bürk et al., 2004), but before presenting it, other SCAs and their genetic causes will be discussed. All of the discussed SCAs will be referred to later on in this thesis. Spinocerebellar ataxias (SCAs), originally described as autosomal dominant cerebellar ataxias (ADCAs) by Greenfield (1954) and later by Harding (1982) and Koeppen and KD (1984), are a heterogeneous group of genetically and symptomatically separate ataxias (Manto, 2005). SCAs are progressive neurodegenerative conditions that affect the cerebellum and its afferent and efferent pathways (Taroni and DiDonato, 2004) as well as spinal cord and brainstem (Schöls et al., 2004). The age of onset varies significantly, however, the average is the third decade of life (Manto, 2005). SCAs have been classified into subtypes, over 40 to date, and are numbered in order of gene discovery (Harding, 1982). SCA occurrence is estimated to be 1 per 100,000 people (Manto, 2005; Ruano et al., 2014), however the frequency differs in various regions, e.g. in Valle d’Aosta Region, Italy, the SCA prevalence is 14.8/100,000 (Leone et al., 1995), 6.4/100,000 in Portugal (Silva et al., 1997) and 3/100,000 in the Netherlands (van de Warrenburg et al., 2002).

In some areas certain types of SCAs are recorded at unusually high frequencies, potentially indicating a founder effect. A founder effect is observed when only a few in-
individuals or a single pair establish a new population in a separate geographical location (Mayr, 1942). For example, although SCA3 is the most common subtype worldwide, SCA1 is most commonly diagnosed in Australia (Storey et al., 2000) and Poland (Krysa et al., 2016), SCA7 in Veracruz, Mexico, (Magaña et al., 2014) and SCA2 in Holguín, Mexico, (Velázquez Pérez et al., 2009).

The clinical presentation of SCAs has a wide range of neurological symptoms, such as ataxic gait, dysarthria, oculomotor deficits, cognitive impairment and epilepsy (Schöls et al., 2004). Ataxia and dysarthria are observed in all SCAs, however the presence of other symptoms is more specific to different SCA subtypes (Zoghbi and Orr, 2000). A wide overlap between symptoms of different SCAs as well as phenotypic differences in affected family members present difficulties in SCA diagnosis, which is usually achieved by genetic testing (Schelhaas et al., 2000; Schöls et al., 2004; Manto, 2005). SCAs also differ in age of onset, symptom progression and severity.

**Poly-glutamine expansion SCAs**

Poly-glutamine expansion resulting in an elongated polyQ tract has been identified as the underlying genetic cause in SCA 1, 2, 3, 6, 7 and 17 (Imbert et al., 1996; David et al., 1997; Zhuchenko et al., 1997; Nakamura, 2001) and accounts for half of dominant ataxia cases (Sailer and Houlden, 2012). In these ataxias genetic anticipation is observed with the number of repeats increasing with each generation in turn increasing the severity of ataxic symptoms (Sailer and Houlden, 2012). It has also been observed that with more copies of repeats and earlier disease onset, the specificity of neuronal loss and systems affected seem to diminish (Zoghbi and Orr, 2000). For example, young SCA1 patients also demonstrate cognitive abnormalities alongside cerebellar and brainstem dysfunction, while in SCA7 the heart undergoes degeneration together with neuronal loss (Zoghbi and Orr, 2000).
SCA1

SCA1 was the first SCA of which the underlying genetic abnormality was established (Orr et al., 1993; Banfi et al., 1994). In SCA1 degeneration of Purkinje cells as well as neurons in inferior olive, dentate nucleus and cranial nerve nuclei is observed (Zoghbi and Orr, 2000). Orr and colleagues discovered multiple repeats of CAG trinucleotide, coding for glutamine amino acid, in the Ataxin-1 gene (Orr et al., 1993). They also established a correlation between the number of repeats and the age of symptom onset (Orr et al., 1993). Further studies have shown that mutant ataxin-1, which like normal ataxin-1 localises to the nucleus, forms inclusions (Skinner et al., 1997). It has also been demonstrated that the polyQ expansion favors the protein complex containing RNA Binding Motif Protein 17 (RBM17) and attenuates the formation of complex with capicua, therefore gain-of-function and partial loss-of-function both contribute to the neuropathology of SCA1 (Lim et al., 2008).

SCA2

SCA2 is the second most common SCA type in the world (Schöls et al., 2004), with 5-7 cases per 100, 000 people worldwide (Velázquez-Pérez et al., 2011). The patients usually exhibit dysarthria and ataxic gait (Velázquez-Pérez et al., 2011) and are diagnosed around 30 years of age (Velázquez-Pérez et al., 2011). A fifth of patients also show symptoms of motor neuron involvement (Berciano, 2007). Furthermore, SCA2 symptoms include cognitive disturbances and sleep abnormalities (Velázquez-Pérez et al., 2011). As some patients have very large CAG expansions, they also demonstrate quite rare SCA2 symptoms like myoclonus-epilepsy (Tan et al., 2004) and retinitis pigmentosa (Babovic-Vuksanovic et al., 1998).

The genetic abnormality underlying SCA2 was identified by Imbert et al. (1996) which was multiple repeat CAG expansion in the ATXN2 gene. The gene codes for the ataxin-2 protein, which is ubiquotously expressed (Imbert et al., 1996). The function of ataxin-2 is still not clearly understood, however, some researchers proposed an involvement in RNA metabolism (Shibata et al., 2000) and plastin-association pathways.
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(Ralser et al., 2005).

Of note, some studies have identified a link between SCA2 and Amyotrophic lateral sclerosis (ALS). It has been shown that 34 or more CAG repeats in ATXN2 are found in SCA2 patients (Pulst et al., 1996), while intermediate expansion of CAG repeats (27 to 33) are found in ALS patients (Elden et al., 2010; Van Damme et al., 2011). This occurs due to TAR DNA-binding protein 43 (TDP-43) interaction with ataxin-2, suggesting that ATXN2 could be one of the underlying genes in ALS (Elden et al., 2010).

SCA3

Out of all SCAs, SCA3, also known as Machado-Joseph disease, is considered the most common (Schöls et al., 2004; Manto, 2005; Ruano et al., 2014) representing 20% of dominant ataxias in the US (Paulson, 2012). Not much neuronal degeneration is observed in the cerebellum of individuals with SCA3, with biggest neuronal loss in basal ganglia, spinal cord and brainstem (Zoghbi and Orr, 2000). The disease is brought about by CAG trinucleotide expansion (52-86 copies) in the ataxin-3 gene (Kawaguchi et al., 1994; Schöls et al., 1995; Todd and Paulson, 2010) and instability of the CAG repeat number has been reported with correlation of symptom presentation (Manto, 2005). Maruyama et al. (1995) and Kawaguchi and Hirano (2013) found a negative correlation between number of repeats and age of onset. The disease neuropathology studies have shown that the cells expressing mutated ataxin-3 undergo apoptosis (Ikeda et al., 1996), while a study by Chai et al. (1999) showed that proteosome - ubiquitination pathways are strongly implicated in SCA3.

Non-coding repeat SCAs

Non-coding repeats have also been found to be the underlying genetic abnormalities in others SCAs, for example SCA8 (CUG repeats) (Koob et al., 1999) and SCA12 (Holmes and O’Hearn, 1999). Other types of expansions have also been found in various SCAs - ATTCT repeat expansion was identified in SCA10 (Matsuura et al., 2000), TGGAA
Conventional genetic mutations

In other SCAs conventional genetic mutations have been identified as underlying causes as reviewed by Perkins et al. (2016b), however, each subtype is generally quite rare with just few families carrying the mutation (Sailer and Houlden, 2012). Patients with conventional mutations usually have a milder clinical phenotype with slower disease progression (Sailer and Houlden, 2012). Nevertheless, it is estimated that conventional mutations account for 40% of still unidentified genetic causes for ataxia, and reducing costs of genome sequencing methods will help to identify previously unknown genes (Sailer and Houlden, 2012).

SCA28

SCA28 was identified in a four-generation Italian family (Cagnoli et al., 2006). The age of onset was determined to be around 20 years of age and patients demonstrate ataxic gate, incoordination and speech difficulties (Cagnoli et al., 2006). The underlying mutation was identified in a gene, encoding mitochondrial metalloprotease AFG3-like matrix AAA peptidase subunit 2 (AFG3L2), which shows a strong expression in cerebellar Purkinje cells (Di Bella et al., 2010). The protein is expected to play a role in quality-control (Tatsuta and Langer, 2008) and synthesis of mitochondrial proteins (Esser et al., 2002). Expressing mutant AFG3L2 in yeast showed affected respiration (Di Bella et al., 2010). A study by Maltecca et al. (2015) demonstrated that mice heterozygous for mutant AFG3L2 protein had abnormally high calcium levels and also led to dark cell degeneration.

Niemann-Pick Disease type C

Named after Albert Niemann and Ludwig Pick, Niemann-Pick disease type C (NPC) encapsulates heterogenous lyposomal lipid storage disorders (Vanier, 2010). The underlying disease causes were identified to be mutations in genes NPC1 (Carstea et al.,
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1997) and NPC2 (Naureckiene et al., 2000). The exact functions of the proteins are not yet clear, however they are suggested to be involved in cholesterol and other molecule transport in the cell (Sleat et al., 2004; Ioannou, 2005). The age of disease onset is very varied from prenatal day to adulthood (Vanier, 2010) and the patient survival ranges from a few days to decades (Vanier, 2010). The disease affects a range of organ systems with visceral involvement as well psychiatric and neurological abnormalities (Vanier, 2010). In adult NPC cases, ataxia and dysarthria are also observed (Vanier, 2010).

1.4 SCA5 and SPARCA1

SCA5

SCA5, which is the main focus of this thesis, was first identified in 1994 by Ranum et al. (1994) by examining 170 individuals from a 10 generation family. The symptoms of SCA5 are described as more benign with clinical signs indicating cerebellar abnormalities and little evidence of other system involvement (Holmes, 1907; Ranum et al., 1994). SCA5 symptoms are ataxic gait, slurred speech, upper extremities incoordination and nystagmus (Ranum et al., 1994; Stevanin et al., 1999). Coordination, gait as well as articulation get progressively worse with patients requiring a wheelchair years after disease onset (Liquori et al., 2001). Brain images of patients reveal degeneration in cerebellum.

Non cerebellar signs have also been observed in isolated families, such as myokynia, which is described as spontaneous muscle contractions without any observed muscle weakness or atrophy. On average, the symptoms appear between 30 and 40 years of age, however in some patients it has been observed to appear as early as 10 and as late as 68 years (Ranum et al., 1994). Adult-onset SCA5, however does not appear to shorten the expected lifespan (Ranum et al., 1994). Bulbar paralysis, suspected to play a role in recurrent pneumonia (Zoghbi, 1991), is only observed in young-onset SCA5 cases.

A second family, in which SCA5 was prevalent, was identified in 1999 by Stevanin
et al. (1999). Magnetic resonance imaging of one of the patients in this study showed that marked cerebellar atrophy of vermis and hemispheres is present, however the patient was experiencing only mild ataxia (Fig. 1.5). Post-mortem analysis of SCA5 patient brains confirmed extreme loss of Purkinje cells and thinning of the molecular layer (Liquori et al., 2001). Some loss of granule cells was also observed (Liquori et al., 2001). Some studies have suggested anticipation in SCA5 presentation, i.e. later generations showing SCA5 symptoms earlier (Ranum et al., 1994; Liquori et al., 2001), but these findings have not been significant and the possible mechanism underlying it is currently unknown. The third family to show SCA5 phenotype was from Germany, and affected family members presented with nystagmus in addition to previously described SCA5 symptoms (Bürk et al., 2004).

![Figure 1.5: MRI image of SCA5 patient illustrating cerebellar atrophy. The patient has been experiencing mild cerebellar symptoms for 4 years. Courtesy of Stevanin et al. (1999)](image)

**Mutations underlying SCA5**

In all three families, the genetic locus was identified as chromosome 11 (11q13) (Ranum et al., 1994; Stevanin et al., 1999; Bürk et al., 2004). In 2006 a study by Ikeda et al. revealed that the mutations underlying the SCA5 in all three families are located in *SPTBN2* gene. The American family possess a 39-bp deletion in exon 12, which in turn
results in a 13 amino acid in-frame deletion within the third spectrin repeat. In the French family, a 15-bp deletion in exon 14 was detected also resulting in an in-frame 5 amino acid deletion in the third spectrin repeat. Finally, the German family had a point mutation in exon 7. This mutation resulted in a leucine to proline change within the highly preserved actin binding site (Ikeda et al., 2006). Since then, new mutations have been reported in family pedigrees where ataxia was present. A study by Cho and Fogel (2013) detected threonine to methionine substitution in second β-III spectrin repeat in a highly conserved region. Wang et al. (2014) found a glutamine deletion in the sixth spectrin repeat, potentially effecting the protein structure. All of the above mutations, identified in \textit{SPTBN2} were heterozygous.

\textbf{Spectrin-associated Autosomal Recessive Cerebellar Ataxia type I (SPARCA1)}

In 2012 a homozygous recessive premature nonsense mutation (Fig. 1.6) was found in the \textit{SPTBN2} gene in individuals affected with a new genetic condition Spectrin-associated Autosomal Recessive Cerebellar Ataxia type I (SPARCA1), suggesting that β-III spectrin mutations might be implicated in more forms of ataxia than previously thought (Lise et al., 2012). Furthermore, individuals with SPARCA1 are cognitively challenged in addition to possessing motor deficits (Lise et al., 2012), indicating various roles for β-III spectrin in normal brain function. In this study the age of onset was significantly earlier - at one year of age - with global developmental delay. The cerebellar atrophy was also found to be progressive when the same patient was imaged at 6 and 15 years of age (Fig. 1.7).

\textbf{Figure 1.6:} Diagram of mature spectrin protein with some known mutations in \textit{SPTBN2} gene. Adapted from Lise et al. (2012).
Other studies have since reported new mutations causing infantile-onset ataxia with cognitive and developmental abnormalities. Arginine to tryptophan substitution (R480W), a heterozygous mutation in the second spectrin repeat (Jacob et al., 2013; Parolin Schnekenberg et al., 2015), as well as a homozygous 5 bp deletion (c.2864_2868del) identified by Elsayed et al. (2014), both are predicted to affect spectrin folding resulting in expression of a truncated protein.

**Figure 1.7:** MRI image of the same SPARCA1 patient at 6 (left) and 15 (right) years of age demonstrating progressive degeneration of the cerebellum. Courtesy of Lise et al. (2012).

**Varied presentations of mutations in the same gene**

In most SCA5 cases patients with heterozygous mutations have late onset and relatively mild ataxia while homozygotes suffer from infantile-onset ataxia with cognitive impairment, suggesting that mutations in the same gene have variable presentation. Such observations have been previously made in other SCAs caused by mutations in 1,4,5-trisphosphate receptor type 1 gene (ITPR1), potassium voltage-gated channel subfamily c member 3 (KCNC3) and fibroblast growth factor 14 (FGF14) as reviewed in Perkins et al. (2016b), however, the molecular mechanisms of it remains unclear.
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1.5 Mouse models of ataxia

Animal models mirroring the symptoms of hereditary ataxias have helped to further cerebellar research (Manto and Marmolino, 2009). As underlying causes for cerebellar ataxia are very varied (Manto, 2005), there are high number of animal models aiming to replicate human condition (Cendelin, 2014). Mice with naturally occurring spontaneous mutations were the first, now known as classic, animal models of ataxia (Cendelin, 2014).

1.5.1 Spontaneous mouse models of ataxia

The very first mouse models of ataxia which were used to pioneer ataxia research have been around for 50 years and were all caused by spontaneously occurring mutations (Lalonde and Strazielle, 2007; Cendelin, 2014). These animal models facilitated ataxia research and allowed investigation of disease pathology (Cendelin, 2014). The animal models introduced in this section are referred to later on in this thesis or have similar disease mechanisms as discussed later on.

A lot of classic ataxia mouse models have similar disease pathogenesis. In lurcher, Purkinje cell degeneration (pcd), nervous, staggerer (sg), reeler and scrambler mice Purkinje cell degeneration and ectopic Purkinje cells are observed. Neurodegeneration of other cell types like granule cells in scrambler and weaver mice ataxia models have also been observed as a potential outcome of Purkinje cell death. In contrast, in weaver mice the abnormalities in granule cell migration results in altered cerebellum architecture and misplaced Purkinje and granule cells. These findings show that although Purkinje cell abnormalities are in the centre of ataxia pathology, these alterations are not necessarily cell-intrinsic and other cell malfunction can also be causal.

In both, the lurcher and hotfoot mouse models, glutamate signaling has been observed to be affected altering cell excitability. Similarly, mutations in G-protein coupled inwardly rectifying potassium channels (GIRK2) in weaver mice underlies an ataxic phenotype, suggesting that altered cell excitability is implicated in several ataxic models.

Depending on underlying genetic causes, neurodegeneration in other brain regions
was also reported. For example, in lurcher mice the hindbrain is affected, while in pcd mouse model bulbar involvement is observed, highlighting variability in ataxia-accompanying symptoms.

**Lurcher**

Lurcher mice were first identified and reported by Phillips (1960) and exhibit ataxia, stumbling and abnormal locomotion (Fortier et al., 1987) with poor performance on a rotarod (Caston et al., 1995b) and static wooden beam (Le Marec et al., 1997). The dominant mutation in the Grid2 gene, encoding δ2 glutamate receptor (GluRδ2) underlies the gain-of-function disease mechanism (Zuo et al., 1997). The normally non-functioning channel expression is found in Purkinje cells (Araki et al., 1993). Mutant GluRδ2 receptor, however, demonstrates enhanced potency to glutamate by 2, 000 fold turning GluRδ2 receptor into a functioning channel (Taverna et al., 2000) and increases the inward Na⁺ current (Zuo et al., 1997). Such shift results in a depolarised membrane potential of Purkinje cells (Zuo et al., 1997). Homozygous mice do not survive long after birth due to huge neuronal loss in the hindbrain (Cheng and Heintz, 1997). Heterozygous mice survive, however Purkinje cell degeneration starts at postnatal day 8 (P8) and none of the Purkinje cells survive beyond P90 (Caddy and Biscoe, 1979). A study by Duffin et al. (2010) demonstrated that the anterior regions of the cerebellum are the ones to lose Purkinje cells first, followed by posterior cerebellum. Granule cell loss follows Purkinje cell death (Wetts and Herrup, 1982).

**Hotfoot**

Recessive mutations causing deletions in the same Grid2 gene, which underlies lurcher mouse phenotype, were reported in hotfoot mouse (Lalouette et al., 1998, 2001). This loss-of-function mutation causes mutant GluRδ2 to be retained in the endoplasmic reticulum (ER) without reaching the cell surface (Matsuda and Yuzaki, 2002). This results in a loss of presynaptic denervation parallel fibre-Purkinje cell synapse (Guastavino et al., 1990; Liu and Shio, 2008). Mice demonstrate mild cerebellar ataxia, hopping phe-
notype and wide base (Guastavino et al., 1990). Hotfoot mice also show significantly poorer performance in a rotarod test (Lalonde et al., 2003a).

**Purkinje cell degeneration (pcd)**

Recessive mutations in the *Agtpbp1* gene (Mullen et al., 1976), which encodes ATP/GTP binding protein 1 (Fernández-González et al., 2002) underlie the phenotype of pcd mice. Normally, mRNA of ATP/GTP binding protein 1 is detected in Purkinje cells, photoreceptors in the retina, olfactory bulb mitral cells and some thalamic neurons (Fernández-González et al., 2002). In the pcd mice, Purkinje cell degeneration is observed between third and fourth postnatal weeks with rapid progression (Mullen et al., 1976). Granule cell degeneration, following Purkinje cell death, has also been noted (Ghetti et al., 1987; Triarhou, 1998). Outside of the cerebellum, the number of photoreceptors start to degenerate by the third postnatal week and are all the gone within a year (Mullen and Lavail, 1975; Blanks et al., 1982). Neurodegeneration in the mitral cells is observed in the olfactory bulb by the second month and only 4% of cells are found at 8 months of age (Greer and Shepherd, 1982). In the ventral geniculate nucleus of the thalamus, by P90 a small proportion (~9%) of neurons are found to have degenerated (O’Gorman, 1985). Ataxic symptoms of pcd mice appear around the same time as Purkinje cell degeneration is taking place (3 weeks of age) (Mullen and Lavail, 1975). Ataxic symptoms are not obvious at this age, but progressively worsen over the next few weeks (Mullen and Lavail, 1975) as shown by performance on the rotarod test (Le Marec and Lalonde, 1997).

In addition, by P300, heterozygous pcd mice demonstrate approximately 20% Purkinje cell loss (Doulazmi et al., 2002; Díaz et al., 2012).

**Nervous**

The mutation underlying the nervous mouse phenotype has not yet been identified, but the *nr* locus has been mapped to chromosome 8 (Campbell and Hess, 1996; De Jager et al., 1998). The most prominent feature of nervous mice is selective degeneration of 90%
of Purkinje cells which occurs between P23 and P50 with only 10% of Purkinje cells surviving into adulthood (Landis, 1973). Retrograde degeneration in 30% of inferior olive neurons was also described (Zanjani et al., 2004). The phenotype of nervous mice is hyperactivity and ataxia (Lalonde et al., 2003a). The mutant mice perform significantly worse on a rotorod when compared to age matched WT animals (Lalonde et al., 2003a). Nervous mice also undergo degeneration in their retina with 30% of photoreceptors gone by 3 weeks of age (LaVail et al., 1993) as well as dorsal cochlear nucleus (Berrebi and Mugnaini, 1988).

**Staggerer (sg)**

Sg mice have an autosomal dominant deletion in ROR2 which belongs to retinoid-related nuclear hormone-receptor superfamily and results in the ligand-binding domain not being translated (Hamilton et al., 1996). This receptor is widely expressed in Purkinje cells (Nakagawa et al., 1997; Ino, 2004) and is suspected to play a role in neuronal maturation (Hamilton et al., 1996). Sg mice are described to have small body size, hypotonia, tremor and staggering gait (Sidman et al., 1962). The development of Purkinje cell spines has been shown to be delayed and dendritic branchlet extension is only detected at P23 (Hirano and Dembitzer, 1975). Purkinje cell numbers were also reported to decline before P5 and by the end of the postnatal week 4, 75% of Purkinje cells were found to have degenerated (Herrup and Mullen, 1979a). The surviving Purkinje cells were also found to have significantly reduced soma size and smaller dendrites (Sidman et al., 1962; Landis and Sidman, 1978). Purkinje cell degeneration was reported to vary regionally, with cerebellar mid-line and lateral cerebellum preserving most Purkinje cells. In the intermediate regions just few Purkinje cells were detected as well as absence of cerebellar folia folding (Herrup and Mullen, 1979b). The remaining Purkinje cells were reported to be misaligned (Sidman et al., 1962) and granule cell death was also observed (Herrup, 1983). Sg mice perform significantly worse on a rotorod test than their age-matched controls (Caston et al., 1995a). Of note, the use of sg-WT mice chimeras has demonstrated that cell abnormalities in sg mice are cell-intrinsic, as the
level of degeneration was between sg heterozygous and homozygous mice (Herrup and Mullen, 1979b).

**Weaver**

The autosomal semi-dominant G to A substitution in *Girk2* gene, coding for GIRK2, underlies weaver mouse phenotype (Patil et al., 1995). GIRK2 is widely expressed in the brain and in WT mice it has been found to have the strongest expression in the granular layer. In contrast, only a few granule cells in weaver mice express GIRK2 with mostly Purkinje cells showing GIRK2 expression (Lauritzen et al., 1997). The cerebellar anatomy of weaver mice has been reported to be significantly altered, with absence of granule cells, but Purkinje cells surviving and possessing dendritic trees, however, they were misaligned (Hirano and Dembitzer, 1973; Rakic and Sidman, 1973). The granule cells have been shown to fail their migration to internal granular layer, resulting in subsequent neuronal death (Rakic and Sidman, 1973). The mutation is expected to alter cell excitability, which affects granule cell differentiation (Patil et al., 1995). Mild Purkinje cell loss has been observed in homozygous and heterozygous weaver mice with 72% and 86% of Purkinje cells surviving (Blatt and Eisenman, 1985). In homozygous weaver mice, Purkinje cells have been found to be misaligned and their dendritic trees were observed to be randomly oriented (Rakic and Sidman, 1973). Verapamil, a calcium channel blocker, has been successfully used to restore normal granule cell migration (Kofuji et al., 1996). Unexpectedly, GIRK2 KO mice had no cerebellar abnormalities, suggesting gain-of-function effect in weaver mice (Signorini et al., 1997). The mice perform poorly on the rotarod (Strazielle et al., 2006).

**Reeler**

An autosomal recessive mutation in the gene coding for reelin protein, which plays a role in neuronal cell migration, was identified to underlie the reeler mouse phenotype (D’Arcangelo et al., 1995). Reelin is mostly expressed in granule cells (Schiffmann et al., 1997), therefore granule cell loss is observed in these mice (Caviness and Ra-
Reeler mice cerebellar architecture has been observed to be significantly altered, with displaced Purkinje cells, reduced granular layer and cerebellar volume (Hamburgh, 1963; Mariani et al., 1977). Other brain regions have also been reported to be affected, such as inferior olive, hippocampus and neocortex (Cendelin, 2014). Reduction in Purkinje cell number was reported with less than 50% of Purkinje cells surviving, and only 5% of them are found in their normal position (Heckroth et al., 1989). Furthermore, each surviving Purkinje cell was found to receive innervation of multiple climbing fibres, rather than one (Mariani, 1982). All of these findings are result of disturbed cell migration (Pinto-Lord et al., 1982; Hack et al., 2002). Reeler mice have symptoms of ataxia and perform poorly in a rotarod test (Lalonde et al., 2004).

Scrambler

Scrambler mice were first identified and described in the study published by Sweet et al. (1996). Scrambler mice have an autosomal recessive mutation in the gene coding for disabled-1 protein resulting in ataxic gait and tremor which appears at P8. Their cerebellum also lacks folia (Sweet et al., 1996; Jacquelin et al., 2012). Similarly to reeler mice, scrambler mice lack approximately 80% of granule cells and have reduced Purkinje cell numbers, of which only 5% are found to be located between molecular and granular layers (Goldowitz et al., 1997). Scrambler mice also perform significantly worse on a rotarod than their age matched WT control mice (Jacquelin et al., 2013). Scrambler mice show similar phenotype to reeler mice, probably due to disabled-1 protein being within the reelin protein signaling cascade (Goldowitz et al., 1997).

1.5.2 Transgenic mouse models of ataxia

As more and more genetic causes were identified underlying various SCAs, mouse models introducing similar mutations or genetic abnormalities into the same genes as the affected genes in patients were produced (Burright et al., 1995; Huynh et al., 2000; Watase et al., 2002; Aguiar et al., 2006; Lalonde and Strazielle, 2007; Perkins et al.,
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2010; Stankewich et al., 2010; Hansen et al., 2013; Armbrust et al., 2014; Cendelin, 2014). The mouse models are aimed to mimic clinical features of ataxia patients (Lalonde and Strazielle, 2007) and multiple mouse models are available in some SCAs. Experimenters, therefore, have to make an informed choice before using any of them to address different questions in disease pathophysiology (Cendelin, 2014). Transgenic mouse models introduced in this section are referred to later on in this thesis.

### 1.5.3 SCA1

The first SCA1 transgenic mouse model was generated by Burright et al. (1995). The authors introduced the human ataxin-1 DNA sequence under the Purkinje cell protein 2 (Pcp2) promoter into mice without and with 82 CAG repeats. While the Purkinje cells with non-expanded ataxin-1 gene were normal, the Purkinje cells expressing the expanded gene version showed Purkinje cell degeneration and abnormal Purkinje cell trees (Burright et al., 1995). The ataxic symptoms and abnormal gait were obvious at 12 weeks of age (Burright et al., 1995), but these mice showed a worse performance on the rotarod test at 5 weeks of age (Clark et al., 1997). Lin et al. (2000) reported that in SCA1 mice even before the disease pathology is observed, downregulation of genes responsible for signal transduction and calcium homeostasis has occurred. Furthermore, levels of calbindin D28k and parvalbumin were significantly lower in Purkinje cells, implicating calcium signaling in SCA1 pathology (Vig et al., 1998).

Another mouse model was introduced in 2002 by Watase et al. and relied on a knock-in approach where a human 74 CAG repeat mutation was introduced into the SCA1 disease locus into mouse genome. The ataxia phenotype was observed by 20 weeks of age with none of the mutant animals surviving beyond 50 weeks of age.

### 1.5.4 SCA2

The first transgenic mouse model of SCA2, carries 58 CAG repeats in the introduced human ataxin-2 gene with Pcp2 promoter (Huynh et al., 2000). In this mouse model, 50% of Purkinje cells degenerate by 24-27 weeks of age. Furthermore, the mouse gait is
altered with significantly shorter steps recorded at 8 weeks of age (Huynh et al., 2000). The ataxia has also been observed to be progressive, with significantly shorter times on a rotarod at 16, but not 6 weeks of age (Huynh et al., 2000). An alternative mouse model, which has 75 CAG repeats by Aguiar et al. (2006), showed earlier disease onset in the homozygotes rather than heterozygotes.

In a mouse model by Hansen et al. (2013), a decrease in calcium regulating protein, calbindin, and Pcp2 has also been observed. This gene dysregulation predates motor deterioration, which is already observed at 8 weeks of age on an accelerating rotarod (Hansen et al., 2013). Between different mouse lines, the severity of the symptoms was proportional to the number of CAG repeats in the polyQ expansion (Cendelin, 2014), similarly to observations in human patients (Riess et al., 1997). Purkinje cells in SCA2 mice were also more sensitive to glutamate signaling, causing cell death (Liu et al., 2009).

### 1.5.5 SCA5

Three separate groups have produced SCA5 mouse models Stankewich et al. (2010); Perkins et al. (2010); Armbrust et al. (2014). In this section of the thesis similarities and differences between these mouse models, as well as different approaches to generating transgenic animals, will be discussed.

**Spnb3**<sup>-/-</sup> by Stankewich et al. (2010)

Mutations in *SPTBN2* gene have been demonstrated to underlie SCA5 (Ikeda et al., 2006). In March 2010, a study by Stankewich et al. reported a mouse model of SCA5. A Spnb3<sup>-/-</sup> mouse was generated by inserting β<sub>geo</sub> casette between exons 25 and 26. The gene insertion resulted in a truncated β-III spectrin protein terminating with spectrin repeat 14. It was observed that in Purkinje cells of KO mice β-III spectrin localised in the membrane and cell some forming aggregates. Shrinking Purkinje cells were also detected with 15-20% thinner molecular layer, however, no Purkinje cell loss was reported even at 1.5 years of age. In KO Purkinje cell culture, swelling of endoplasmic
reticulum and Golgi complexes was also observed.

As Jackson et al. (2001) has reported before, β-III spectrin stabilises EAAT4 at the cell membrane. Not surprisingly, levels of EAAT4 were significantly reduced in Spnb3−/− Purkinje cells, but no loss was observed for Ca2+/calmodulin-dependent protein kinase II α (CamKIIα). The level of GluRδ, however, was significantly smaller.

Spnb3−/− mice were also reported to perform significantly worse on a rotarod, demonstrating mild ataxia and gait abnormalities by 8 months of age. Unexpectedly, myoclonic seizures were also recorded in these mice, the frequency of which increased with age. The seizures were observed to be induced by handling and disturbing mice. Heterozygotes showed slight motor impairment as well.

**β-III−/− by Perkins et al. (2010)**

A month after publication by Stankewich et al. (2010), another study (Perkins et al., 2010) reported a different mouse model for SCA5. This was a β-III spectrin KO mouse model generated by targeted recombination, which replaced exons 3-6 with neomycin cassette. This insertion disrupted the open reading frame, by splicing exon 2 onto exon 7 and introducing a premature stop codon at exon 7. However, a form of β-III spectrin was still found to be expressed at low levels throughout the dendritic tree of Purkinje cells, resulting from exon 1 being spliced onto exon 7. This form of β-III lacks majority of actin binding site.

Phenotypically β-III spectrin KO mice were found to develop progressive ataxia. The gait of KO mice was found to get wider with age suggesting an unstable gait. In a rotarod task, young KO mice performed significantly worse only at higher speeds (10 rpm), however by 6 months age, they were falling off even at slow speeds (3 rpm), suggesting a slowly progressive ataxic phenotype. This was also confirmed by their poor performance at older age on an elevated beam, with more hind-limb slips observed. Older mice also had tremor, but exhibited normal cage behaviour even at old age.

Histological analysis revealed significant thinning of the molecular layer. By 6 months of age, 20% decrease in PC number was also observed together with dark,
shrinking cell bodies, associated with excitotoxicity induced neuronal death (Garthwaite and Garthwaite, 1991). Significant degeneration of Purkinje cell dendritic trees was later reported in a study by Gao et al. (2011) using the same mouse model.

Similarly to the study by Stankewich et al. (2010) and in line with EAAT4 reduction in SCA5 patients found during autopsy, a significant drop in EAAT4 levels was observed in β-III spectrin KO mice.

Electrophysiological findings revealed that Purkinje cell spontaneous firing of simple spikes was reduced, with smaller frequencies observed at 12 weeks and 8 months of age, when compared to WT mice. Additionally, sodium currents in acutely dissociated Purkinje cell neurons were significantly smaller in KO cultures, implicating sodium channel abnormalities in SCA5. Analysis of excitatory inputs from parallel fibre onto Purkinje cells, the parallel fibre excitatory post-synaptic currents (EPSCs) were found to be significantly enhanced in young β-III spectrin KO mice.

In contrast to the study by Stankewich et al. (2010), no abnormalities were observed in β-III spectrin heterozygous mice (Clarkson et al., 2010), supporting the hypothesis that SCA5 is brought about not by haploinsufficiency, but dominant negative effect of the mutated β-III spectrin. This also suggests that heterozygote ataxia observed by Stankewich et al. (2010) is possibly brought about by a dominant negative effect of the expressed mutant protein.

Δ39 β-III spectrin by Armbrust et al. (2014)

Armbrust et al. (2014) have recently published a novel SCA5 mouse model in which a conditional transgenic mouse was generated by introducing human β-III spectrin containing the reported American mutation (Ranum et al., 1994). In this mouse model EAAT4 protein levels were not found to be reduced, in contrast to animal models produced by Stankewich et al., Perkins et al. as well as SCA5 patient autopsy tissue (Ikeda et al., 2006). β-III spectrin mice were found to perform normally on the rotarod at 6 weeks of age, but showed signs of progressive ataxia at 26 weeks of age. Thinning of molecular layer was also observed, suggesting Purkinje cell dendritic tree degeneration.
This study also found that β-III spectrin and metabotropic glutamate receptor type 1 α (mGluR1α) can be co-immunoprecipitated, and the binding site on β-III spectrin was identified as being within spectrin repeats 14-16. However, the presence of the American mutation was not found to disrupt this interaction. Nevertheless, the stabilisation of mGluR1α was reduced at the plasma membrane, and with functional consequences of reduced mGluR1α baseline activity.

1.6 Similar pathologies in mouse ataxia models

Although various SCA types have different underlying genetic causes as well as a variety of affected molecular mechanisms, some similarities can be observed. The following section will discuss similar molecular mechanisms involved in different SCAs.

1.6.1 Calcium signaling

Calcium is a universal signaling molecule, playing a key role in neuronal plasticity (Berridge, 1998; Kawamoto et al., 2012). It has been demonstrated to regulate dendritic formation (Lohmann and Wong, 2005), gene transcription (Redmond and Ghosh, 2005), synaptic vesicle release (Südhof, 2004) and membrane excitability (Berridge, 1998; Südhof, 2004). Furthermore, it plays a role in long-term potentiation (Grover and Teyler, 1990) and long-term depression (Bolshakov and Siegelbaum, 1994) formation. As a result, calcium is tightly regulated in the cell, by storing it in the ER (Volpe et al., 1991). Cytosolic calcium is also buffered by calcium binding protein such as calbindin and parvalbulmin, which are present in Purkinje cells (de Talamoni et al., 1993).

Purkinje cells respond actively to calcium concentration changes by altering firing of action potentials (Llinás et al., 1980; Usowicz et al., 1992). A calcium increase in a Purkinje cell is a response to excitatory synaptic inputs from parallel and climbing fibres. Glutamate release at these synapse activates AMPA receptors and causes cell depolarisation as well as activation of voltage-gated calcium channels (VGCC) (Usowicz et al., 1992; Gruol et al., 2012). P/Q VGCCs underlie dendritic spikes in response to
Ca\textsuperscript{2+} concentration increase (Usowicz et al., 1992) as well as neurotransmitter release (Ishikawa et al., 2005), while T-type calcium channels are active at hyperpolarised potentials (Gruol et al., 2012; Isope et al., 2012) and play a role in altering membrane potential of postsynaptic cell.

P-type calcium channel was originally identified in the Purkinje cell soma and dendrites (Llinás et al., 1989), while Q-type channels were found in granule neurons (Randall and Tsien, 1995). Both types of channel contain the core Ca\textsubscript{V}2.1 subunit, which are alternative splice variants of the CACNA1 gene (Randall and Tsien, 1995; Bourinet et al., 1999). A polyglutamine repeat expansion (21-27 repeats) within the CACNA1A gene has been identified in SCA6 patients (Zhuchenko et al., 1997) and a mouse model with the 81 polyglutamine repeats knocked-in (KI) closely mirrors the patient phenotype (Watase et al., 2008). Furthermore, a conditional P/Q type calcium channel KI *purky*, in which the channels are lost within the first month after birth, demonstrates an ataxic phenotype (Mark et al., 2011).

Episodic ataxia 2 (EA2) has also been found to result from mutations in the CACNA1A gene causing haploinsufficiency (Ophoff et al., 1996; Riant et al., 2010). Furthermore, haploinsufficiency of this channel has been suggested to underlie cognitive impairment and autism (Damaj et al., 2015). Spontaneous mutations in CACNA1A gene also underlie the leaner mouse mutant phenotype.

Glutamate also activates mGluR receptors in postsynaptic Purkinje cell membrane at the parallel fibre and Purkinje cell synapse (Knöpfel and Grandes, 2002). mGluR receptors are G protein coupled receptors, and their activity results in activation of phospholipase C pathway (PLC). Following mGluR1 receptor activation, a slow calcium release has been shown from internal calcium stores (Netzeband et al., 1997). MGlurR1 and mGluR2 mutant mice show an ataxic phenotype (Alba et al., 1994; Kashiwabuchi et al., 1995).

Deranged calcium signaling has been observed in various neurodegenerative conditions (Tang et al., 2003; Bezprozvanny, 2009). In field of ataxias, for example, calcium abnormalities were detected in SCA2, where in ATXN2-58Q mouse model (Huynh et al., 2016).
mutant ataxin-2 protein associates with an intracellular Ca\(^{2+}\) release channel and renders it more sensitive to stimuli. As a result, premature Ca\(^{2+}\) release occurs in response to smaller stimuli (Liu et al., 2009). Furthermore, SCA3 mice with 84 polyQ repeats (Cemal et al., 2002) fed with the calcium signaling stabilizer dantrolene showed significantly improved motor performance (Chen et al., 2008). Additionally, large-conductance voltage- and Ca\(^{2+}\)-activated K\(^+\) channel (BK), which can be activated by internal Ca\(^{2+}\) as well a membrane depolarization (Latorre et al., 1989), has been implicated in ataxia since BK KO mice have ataxic phenotype (Sausbier et al., 2004).

### 1.6.2 Glutamate signaling

Enhanced glutamate signaling has been implicated in SCA5. Firstly, Purkinje cells in β-III spectrin KO animals have been observed to undergo dark cell degeneration, which is associated with excessive glutamate signaling (Perkins et al., 2010). This is in line with findings that the EAAT4, normally expressed in the Purkinje cell membrane (Yamada et al., 1996), is stabilised in the membrane at the excitatory synapses only when β-III spectrin is present (Jackson et al., 2001). In β-III spectrin KO mice EAAT4 has been observed to undergo downregulation (Perkins et al., 2010). Blocking of EAAT4 at the synapse has also been shown to increase glutamate synaptic concentration and directly affect AMPA receptor current in Bergmann glia (Tsai et al., 2012) as well as increase the climbing fibre EPSCs (Takahashi et al., 1996). As EAAT4 is an anion channel for chloride anions (Fairman et al., 1995) as well as glutamate transporter, it may also be implicated in Purkinje cell excitability (Tanaka, 2000; Huang and De Schutter, 2011; Perkins et al., 2016a).

Another glutamate transporter, glutamate aspartate transporter (GLAST), is also downregulated in β-III KO mice (Perkins et al., 2010). GLAST is expressed in Bergmann glia (Lehre et al., 1995) and synergistic effects between GLAST, β-III spectrin and EAAT4 have been observed in SCA5 pathophysiology (Perkins et al., 2016a).

Dark cell degeneration has also been observed in ATXN2-58Q mouse model of
SCA2 (Huynh et al., 2000; Liu et al., 2009), as well as SCA28 (Maltecca et al., 2009). SCA5 mouse model by Armbrust et al. (2014) detected smaller mGluR1 activity due to mislocalisation of mGluR1 channels, while in SCA3, mGluR1 mediated-synaptic transmission in parallel fibres was found to be completely abolished, indicating abnormal glutamate signaling (Konno et al., 2014).

1.6.3 Purkinje cell output

Although synaptic input plays a role in determining Purkinje cell firing (Womack and Khodakhah, 2002), Purkinje cells do fire in its absence as well (Häusser et al., 1997). Voltage-gated sodium channels with resurgent current in Purkinje cells are important in determining the intrinsic firing properties of Purkinje cells (Raman and Bean, 1997; Lewis and Raman, 2014). The resurgent current of Na\textsubscript{\textit{v}}1.6 channels is thought to underlie the high-frequency repetitive firing of Purkinje cells (Khaliq et al., 2003). Indeed, mice which lack \textit{Scn8a} gene expression, which encodes Na\textsubscript{\textit{v}}1.6 channels, are ataxic and demonstrate slower Purkinje cell firing frequencies and higher input resistance (Khaliq et al., 2003) when compared to WT Purkinje cells. Reduced sodium currents are observed in \textit{β}-III spectrin KO mice (Perkins et al., 2010) as well as hippocampal neurons expressing \textit{β}-III spectrin mutant constructs (Clarkson et al., 2014). In \textit{β}-III spectrin KO mice stability of Na\textsubscript{\textit{v}}1.1 and Na\textsubscript{\textit{v}}1.6 have been demonstrated to be reduced (Clarkson et al., 2014). In SCA27, which is caused by mutation in \textit{FGF14} gene, sodium currents were also reported to be significantly decreased and Na\textsubscript{\textit{v}} channel α subunit expression was significantly smaller at the axon initial segment (Laezza et al., 2007), causing ataxia.

Other SCAs also demonstrate abnormal Purkinje cell output. For example, in SCA3 significantly more Purkinje cells were found to be silent with depolarised membrane potentials (Shakkottai et al., 2011), while Purkinje cells in SCA1 mouse model with 82 CAG repeats (Burright et al., 1995) were shown to fire at significantly slower frequencies (Hourez et al., 2011).

Calcium signaling abnormalities also have an effect on Purkinje cell intrinsic firing
properties which result in ataxia (Mori et al., 2000; Hoebeek et al., 2005; Walter et al., 2006). For example, a mutation in \( \text{Ca}_{\text{v}2.1} \) \( \text{Ca}^{2+} \) channel in Leaner mouse model has been demonstrated to alter spontaneous action potential timing (Ovsepian and Friel, 2012). Of note, reduction in dendritic tree size was also reported to reduce spontaneous firing in Purkinje cells (Womack and Khodakhah, 2002).

### 1.6.4 Dendritic tree changes

Cellular inputs have been shown to play a key role in Purkinje cell calcium signaling (Kitamura and Kano, 2013) and this activity-driven change in cellular morphology was shown in studies by Rakic and Sidman (1973) and Mason et al. (1997). Glutamate diffusion from parallel fibre presynaptic terminals causes postsynaptic mGluR activation and slow calcium release from intracellular stores (Finch and Augustine, 1998; Takechi et al., 1998). This increase in cytosolic calcium levels is restricted to the area near activated mGluRs, quite possibly underlying dendritic spine plasticity (Nakamura et al., 2004).

Elevated calcium levels have been previously shown to underlie dendritic stabilization (Lohmann and Wong, 2005) as well as intracellular calcium concentration changes have been demonstrated to be involved in dendritic spine formation. Predictably, mice with abnormal calcium signaling have shown abnormalities in the Purkinje cell dendritic tree. For example, mice lacking calbindin, \( \text{Ca}^{2+} \) buffering protein, have significantly smaller dendritic tree spines (Vecellio et al., 2000).

In the ataxia field, the publication by Gao et al. (2011) looked at Purkinje cell morphology in \( \beta-\text{III} \) spectrin KO mice and showed thinning of the dendritic tree, reduced dendritic surface area as well as decreased spine density (Gao et al., 2011). Purkinje cell dendritic tree degeneration is also observed in SCA1 transgenic mice (Burright et al., 1995; Inoue et al., 2001) and SCA3 mouse model, with 69 CAG repeats (Konno et al., 2014). Of note, a study by Womack and Khodakhah (2002) showed that a loss of 2/3 of Purkinje cell dendritic tree directly affected the Purkinje cell spontaneous firing by reducing it, suggesting a close relationship between dendritic tree structure and cell
1.7 Animal model and their use in research

Animal models are widely used to shed light on human disease pathology and have expanded our understanding of various illnesses (van der Worp et al., 2010). New experimental therapeutic approaches are required to demonstrate positive effects in animal studies prior to moving to patients (Sibbald, 2000). Some huge scientific breakthroughs in disease therapies have been achieved due to animal studies. For example, mice model of breast cancer has lead to discovery of trastuzumab, a humanized mouse antibody against human epidermal growth factor receptor 2, which is upregulated in 30% of of human breast cancers (Schwall et al., 2003; Nahta and Esteva, 2007). Animal studies also led to new findings in asthma (Karol, 1994), allowing prediction of safety of new drugs (Shin et al., 2009). Discovery of successful antiviral treatments in HIV has also been facilitated by animal models (Hatziioannou and Evans, 2012).

1.7.1 Animal models of neurological conditions

Mouse models have been invaluable in the study of memory formation (Paul et al., 2009), understanding stroke pathophysiology (Casals et al., 2011; Cruz and Landeira-Fernandez, 2012; Fluri et al., 2015), disturbances in substance addiction (Lynch et al., 2010) and neurodegenerative conditions such as Alzheimer’s (LaFerla and Green, 2012), Parkinson’s (Blesa and Przedborski, 2014) and Huntington’s (Ehrnhoefer et al., 2009). However, only modest successes have been achieved in translating successful results in pre-clinical animal studies to patients, with appropriate animal models being one of the determining factors (van der Worp et al., 2010).

1.7.2 Therapeutic approaches in ataxia and animal models

Despite massive effort and progress in the field, SCAs currently have no cure (Ilg et al., 2014). Various types of therapy, however, are available to patients to help manage the symptoms. For example, controlled studies showed that intensive and frequent phys-
iotherapy sessions improved ataxic symptoms (Miyai et al., 2012). In ataxic children, video game-based coordination training also had positive impact, offering lower cost and highly motivational approaches (Ilg et al., 2012). More studies, though, are required to identify which patients respond best to which kind of physiotherapy, depending on age, disease progression and SCA type (Ilg et al., 2014). Furthermore, usefulness of physiotherapy is questionable in cerebellar degenerative disorders, as cerebellum plays an important role in motor learning (Ilg and Timmann, 2013).

As different types of SCAs are individually quite rare and their underlying genetic causes are quite heterogenous, targeting similar cellular pathways in treatment of different SCAs is thought to be the most promising approach (Ilg et al., 2014). For example, riluzole, which reduces neuronal excitability by opening small conductance potassium channels and reducing glutamate release, have been demonstrated improve dysarthria and motor symptoms of ataxic patients (Ristori et al., 2010). Acetyl-DL-leucine, which normalizes neuronal membrane potential from depolarised and hyperpolarised levels, has also showed promise in improving life quality in SCA1/2 patients (Strupp et al., 2013). Furthermore, amantidine, which is responsible for NMDA receptor blockade, also showed improvement in gait and tremor (Nissenkorn et al., 2013) of ataxia telangiectasia patients. Stimulation of GABA receptors with zolpidem and lamotrigine alleviated atactic symptoms in SCA2 and SCA3 patients, respectively (Clauss et al., 2004; Liu et al., 2005). Unfortunately, most of these clinical studies are performed by pooling patients with different ataxias, making any positive drug effect identification quite complicated (Ilg et al., 2014).

Ataxia has various causes therefore multiple mouse, *C. elegans* and *Drosophila* models are important in mimicking human disease phenotype (Manto and Marmolino, 2009; Cendelin, 2014). The use of such models has led to development of viable therapeutic approaches for ataxia. Friedreich’s ataxia is an autosomal recessive ataxia which is caused by GAA expansion within the gene encoding mitochondrial protein frataxin (Durr et al., 1996). Currently, a clinical trial is underway using EPI-743, which is predicted to protect cells from oxidative stress (Shrader et al., 2011; Enns et al., 2012).
and has shown some promise (Sullivan et al., 2016). In the field of spinorerebellar ataxias, a SCA3 clinical trial is currently ongoing for trehalose, alpha-linked di-saccharide. Trehalose has been shown to allow clearance of mutant ataxin-3 protein and improve motor coordination in a pilot mouse study (Matilla-Dueñas, 2012; Santana et al., 2016).

1.8 Gene therapy

This thesis focuses on gene therapy approach to alleviate the ataxic symptoms, therefore a brief history of gene therapy research will be given. In 1960s scientists started to look at the possibility of introducing foreign DNA into the cells for permanent expression in order to alter their genetic make up (Friedmann, 1992). The first successful experiments showed modest success by identifying, for example inosinic acid pyrophosphorylase (IMPPase)-positive cells from IMPPase negative population following transformation (Szybalska and Szybalski, 1962) or introduction of resistance to 8-azaguanine (Ray Bradley et al., 1962). Such transformations were found to be quite inefficient (Friedmann, 1992), while observations were made that viral transfections yielded high efficiency and stable foreign gene expression (Rogers and Pfuderer, 1968). The first, partially successful mouse study, showed that human globin gene could be introduced into mouse bone marrow using calcium chloride transfection and the transfected cells can repopulate mice bone marrow (Mercola et al., 1980).

Following these promising studies, the following decades saw advances in understanding viral life cycle and biology which made viral vectors an invaluable tool in gene therapy research (Friedmann, 1992) and which holds a lot of promise in single-gene defect hereditary conditions (Koeberl et al., 2009). Gammaretrovirus, lentivirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus are the most commonly used vectors for gene therapy research (Giacca and Zacchigna, 2012) and in 2011, more than 1700 gene therapy clinical trials were being conducted worldwide (Giacca and Zacchigna, 2012). AAVs have been used widely for gene delivery for the last 20 years (Morgenstern et al., 2011) and they are being utilised in 75% of current clinical trials (Lentz et al., 2012; Kumar et al., 2016).
1.8.1 Adeno-associated virus (AAV)

As experiments discussed in this thesis utilised AAV virus, a brief introduction to AAVs will be given. AAVs were first identified by Atchison et al. (1965) and Hoggan et al. (1966). They were found in adenovirus preparations and were initially described as contaminants and defective viruses as they did not cause any known disease in humans (Atchison et al., 1965; McCarty et al., 2004). Due to the requirement for a helper plasmid for viral infection, it took a few years before AAV potential in gene therapy was revisited (Carter, 2004). In the absence of helper plasmids, AAV tend to be quiescent and integrate into chromosome 19 (19q13-qter) at 0.1% frequency (Kotin et al., 1991; McCarty et al., 2004; Deyle and Russell, 2009).

AAVs have a linear single stranded DNA genome, 4.7 kb in length (Russell and Kay, 1999; Daya and Berns, 2008). The viral genome contains two open reading frames for \( rep \) and \( cap \) genes, which encode replication and viral capsid proteins, respectively (McCarty et al., 2004). 145 bp-long inverted terminal repeats (ITRs) flank reading frames and form a characteristic T-shape hairpin structure (Daya and Berns, 2008). ITRs are the only sequences required for in \( cis \) replication and viral genome packaging (Deyle and Russell, 2009).

In genetically engineered AAVs (rAAVs), the transgene of interest is inserted between ITR sequences by replacing \( rep \) and \( cap \) genes (Deyle and Russell, 2009). As a result, for viral production each cell has to be transfected with AAV, carrying the transgene of interest, and helper plasmids encoding \( rep \) and \( cap \) genes (Grimm et al., 1998). The lack of \( rep \) gene prevents rAAVs from integrating into host genome (Afione et al., 1996; Russell and Kay, 1999).

Multiple AAV serotypes, differing by capsid genes, have been identified in humans (Parks et al., 1967) and neutralising antibodies have been detected against them in human serum (Blacklow et al., 1968). AAV2 is the most commonly used AAV serotype for gene therapy (Carter, 2004).

AAV2 vectors transduce the destination cells by binding to the heparan sulfate proteoglycans on the cell surface (Summerford and Samulski, 1998) and the virus is
internalised via clathrin-coated vesicles (Duan et al., 1999). Lowering the pH of these vesicles results in the release of AAV particles (Bartlett et al., 2000).

In order to be of therapeutic use in gene therapy, viral vectors are required to elicit low immune response (Daya and Berns, 2008). Very little immune response is observed following AAV delivery (Zaiss et al., 2002) and the humoral response following AAV delivery is usually due to prior AAV infection (Xiao et al., 1996). Such responses vary from tissue to tissue and are limited in the brain possibly due to blood-brain barrier (Mastakov et al., 2002). However, neutralising antibodies have been identified against AAV2 (Parks et al., 1967; Blacklow et al., 1968; Russell and Kay, 1999; Carter, 2004).

Some AAV serotypes have been shown to transduce neurons (Zhang et al., 2011; Chakrabarty et al., 2013), especially AAV2 (Bartlett et al., 1998), therefore they have been used in research of neurodegenerative conditions such as Parkinson’s (Kaplitt et al., 2007; Björklund and Kirik, 2009), globoid cell leukodystrophy (Lin et al., 2015), ALS (Hester et al., 2009), Alzheimer’s (Lawlor et al., 2007) and Huntington’s (Franich et al., 2008; Southwell and Patterson, 2011). Therapeutic potential of AAVs have also been investigated in SCA1 (Xia et al., 2004), SCA2 (Kasumu and Bezprozvanny, 2012a) and SCA7 (Ramachandran et al., 2014), all of which are discussed in more detail in Chapter 6.

1.9 Aims and approaches

The main aim of my thesis is to investigate the reversibility of SCA5 using a previously published mouse model (Perkins et al., 2010). This will be addressed by firstly investigating cerebellar heterogeneity in SCA5 mice at 12 months of age in Chapter 3, by analysing Purkinje cell morphological and electrophysiological properties in various cerebellar regions. Then, utilisation of primary Purkinje cell cultures will allow investigation of the role of calcium signaling in Purkinje cell tree morphology. This will be achieved by analysing dendritic trees from WT and KO mice in Chapter 4 following application of KN62 and mibefradil, which affect calcium signaling. In the Chapter 5, production of viral particles will be discussed and identification of a promising β-III
fragment will be demonstrated by transfecting Human Embryonic Kidney (HEK293FT) cells and hippocampal neurons. Finally, in Chapter 6 the data will be presented showing the therapeutic effect of partial β-III spectrin delivery by analysing Purkinje cell electrophysiological and morphological properties as well as mice motor performance following viral injection.
Chapter 2

Methods

2.1 DNA subcloning

2.1.1 PCR

Primers for PCR were designed to amplify three fragments of β-III spectrin (Table 2.1 and Table 2.2). Primers incorporated Not I restriction sites to facilitate cloning into mammalian expression vectors pRK5 (Table 2.1) and pL7-mCherry (Table 2.2). Myc-tagged FL β-III spectrin was used as a template for polymerase chain reaction (PCR). For N-trm and C-trm fragments the PCR conditions were: 2 min at 94 °C, followed by 25 cycles of 15 s at 94 °C, 30 s at 65 °C and 2 min at 72 °C with 7 min at 72 °C at the end. For middle fragments and FL β-III spectrin to be cloned into pRK5 and pL7-mCherry vectors, the PCR conditions were: 2 min at 94 °C, followed by 25 cycles of 15 s at 94 °C, 30 s at 65 °C and 7 min at 68 °C and 7 min at 68 °C at the end. PCR products were resolved on a 1% Tris-acetate EDTA (TAE) agarose gel (1% (w/v) agarose (Melford) in TAE (Thermo Scientific) with 0.002% (v/v) ethidium bromide) at 100 V, excised and purified using QIAEXIII Gel extraction kit (Qiagen) following manufacturer’s instructions.
2.1.2 Mammalian expression vectors

Following PCR product purification, half of the DNA was cut with *Not I* at 37 °C for at least 1 hr (10% (v/v) buffer (Promega), 10% (v/v) BSA (Promega), 1 µl 5 U/µl *Not I* (Promega) in dH₂O) followed by purification with QIAEXIII a gel extraction kit (Qiagen). One restriction enzyme unit (U) is the amount of restriction enzyme necessary to completely digest 1 µg of a specific DNA substrate, usually lambda, in 1 hr, in a 50 µl reaction volume and at a specified temperature. In parallel, 2 µg pRK5 and pL7-mCherry vectors were digested with *Not I* (10% (v/v) buffer (Promega), 10% (v/v) BSA (Promega), 1 µl 5 U/µl *Not I* (Promega) in dH₂O) at 37 °C for at least 1 hr. Cut vectors were heat inactivated for 10 min at 70 °C degrees, incubated for 30 min at 37 °C with calf intestinal phosphatase (Promega), resolved on a 1% TAE agarose gel and purified with QIAEXIII Gel extraction kit (Qiagen). DNA ligations were carried out for at least 2 hr at room temperature (RT) and then at 4 °C overnight (10% (v/v) ligation buffer, 10% (v/v) destination vector, 10% (v/v) 1–3 U/ µl T4 DNA Ligase (Promega) and 70% (v/v) DNA insert). DH5α library efficiency cells (Invitrogen) were transformed with 5 µl ligation mixture by heat shocking following manufacturer’s instructions, plated on pre-warmed agar plates (2% (w/v) Luria broth (Sigma), 1.8% (w/v) agar (Invitrogen) and 100 µg/ml ampicillin (Melford)) and kept at 37 °C overnight. Individual colonies were used for inoculation of 2% (w/v) Luria broth (Sigma) and shaken overnight at 37 °C. From overnight cultures plasmid DNA was purified using QIAprep spin Miniprep Kit (Qiagen) following manufacturer’s instructions. The purified DNA was digested with various endonucleases at 37 °C, run on 1% (w/v) agarose (Melfords) TAE (Thermo Scientific) gel at 100 V to investigate the presence and orientation of β-III spectrin fragments. For single and quadruple transfection experiments the following plasmids were used: green fluorescent protein (GFP) expressing, excluded from nucleus due to quintuple lysine repeat insert (Clontech), nuclear yellow fluorescent protein (YFP) (Clontech) and cytoplasmic DsRed (Clontech). They were all a kind gift from Dr Paul Skehel. The fourth plasmid was myc-tagged C-trm β-III spectrin plasmid, already discussed in Section 2.1.1 and Section 2.1.2.
2.1.3 DNA sequencing

60 ng PCR products as well as entry and expression clones from pAAV-Gateway system were prepared in 30 µl dH2O, while the primers were diluted in 10 µl dH2O to 3.2 µM concentration and sent out to DNA sequencing services at the University of Dundee.

2.2 Human Embryonic Kidney cells

Human Embryonic Kidney (HEK293FT) cells were grown in Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich) supplemented with 10% (v/v) heat inactivated foetal bovine serum (hFBS, Gibco), 1 x MEM Non-essential Amino Acid Solution (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma) until they reached ~80% confluence. The cells were split with 0.05% (w/v) trypsin-EDTA solution (Gibco) to ~15% confluence. The day before plating, cover slips were placed into appropriate wells of 6- or 12-well tissue culture plates, washed twice with dH2O and 50 µg/ml poly-L-lysine (Sigma-Aldrich) applied for at least 1 hr at 37 °C. After incubation the cover slips were washed twice with dH2O and air dried prior to plating cells. For immunofluorescence and imaging experiments the cells were split to allow ~10% confluence on the day of transfection and 25-40% confluence in 12-well plates on the day of immunostaining. For experiments comparing various transfection methods, 300,000 HEK293FT cells were plated before calcium phosphate, lipofectamine and X-tremeGENE transfections in a 6-well tissue culture plate. For nucleofection experiments, 700,000 cells were plated on each cover-slip in a 12-well tissue culture. For western blot experiments the cells were split to allow 80-90% confluence in 6-well plates on the day of the experiment. The cells were transfected the next day.

2.2.1 Transfection

X-tremeGENE HP DNA Transfection Reagent (Roche)

Cell medium was replaced 1 hr before transfection. Transfection of a single expression plasmid utilised a 1:1 ratio of µg DNA to µl X-tremeGENE HP DNA transfection
reagent for both immunofluorescence and western blot analysis with 1 µg and 2 µg being transfected, respectively. The mixture was incubated at RT for 20 min before applying the whole mixture volume to desired wells. For co-transfection experiments, the final amount of DNA in a 12-well plate well was 1 µg, of which 0.8 µg was fragment in pL7-mCherry vector and 0.2 µg was pRK5 construct.

In quadruple transfection experiments 2:1 DNA and transfection reagent ratio was used with 0.5 µg of each of the following plasmids: GFP-expressing, excluded from nucleus due to quintuple lysine repeat insert (Clontech), nuclear YFP (Clontech) and cytoplasmic DsRed (Clontech). They were all a kind gift from Dr Paul Skehel. The fourth plasmid was myc-tagged C-trm β-III spectrin plasmid, already discussed in Section 2.1.1 and Section 2.1.2. The transfection efficiency was observed to improve when 2:1 rather than 1:1 ratio of DNA and transfection reagent ratio was used without being toxic to HEK293FT cells.

**Calcium Phosphate**

3 hr prior to DNA transfection, cell media was removed and replaced with pre-warmed 2 ml Iscove’s Modified Dulbecco’s Medium (IMDM, Life technologies), supplemented with 5% (v/v) foetal calf serum (FCS, Gibco). 318 mM RT CaCl$_2$ (Sigma), 162.9 µl dH$_2$O and 0.55 µg of each of the plasmids, used for quadruple-transfection, were mixed together in a 1.5 ml eppendorf and filtered through 0.2 µm filter. Equal volume 2× HEPES-buffered saline (HEBS buffer, Sigma) was added to DNA mix while vortexing vigorously for 15 s and then left to stand for another 1 min and 45 s. The mixture was gently added in a dropwise manner. 16 hr later, the cell media was replaced with 2 ml supplemented MEM described in Section 2.2.

**Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific)**

Transfection was performed according to manufacturer’s protocol. In short, 9 µl Lipofectamine LTX reagent was diluted in 150 µl opti-MEM medium while 2.6 µg DNA (or 0.66 µg of each plasmid in quadruple-transfection experiments) were diluted in another
150 µl opti-MEM with 3 µl PLUS reagent. Two solutions were mixed together and incubated at room temperature for 5 min. 250 µl of the final mixture containing 2 µg DNA was added into cells in 35 mm 6-well tissue culture plate. Ian Monaghan assisted in some of the cell transfection experiments.

**Nucleofection**

HEK293FT cells were nucleofected following Amaxa Cell Line Nucleofector Kit (Lonza) protocol. In summary, after splitting cells as described in Section 2.2 and spinning them at 200× g for 10 min at RT, approximately 1× 10^6 cells were suspended in 100 µl RT nucleofector solution. The suspension was combined with 2 µg DNA and nucleofected using programme Q-001. 70 µl of the mixture was then transferred into a well in a 12-well plate with pre-warmed supplemented HEK cell media from Section 2.2.
### Table 2.1: PCR primer sequences for N-trm, middle and C-trm β-III spectrin fragments to clone into pRK5 vector introducing *Not I* digestion site (underlined).

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<thead>
<tr>
<th>Spectrin fragment</th>
<th>Forward (5'-&gt;3’)</th>
<th>Reverse (5'-&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-trm - SR4</td>
<td>ATTTGCGGCCGCCCATGAGCAGCACCCTGTCACCC</td>
<td>TAAAGCGGCCGCTACTGTGCTAGCCGCTGGGCTCG</td>
</tr>
<tr>
<td>SR2 - SR15</td>
<td>ATTTGCGGCCGCCCATGCAACTGGCTGCTCGCTTCGAC</td>
<td>TAAAGCGGCCGCTAATCCAGTAACAGCTGGCGACG</td>
</tr>
<tr>
<td>SR13 - C-trm</td>
<td>ATTTGCGGCCGCCCATGCTTCGAGCCCAGCAATTCTATCGT</td>
<td>TAAAGCGGCCGCTACTTGTCTTTCTTTAAAAGAAGCTGAATCG</td>
</tr>
</tbody>
</table>

### Table 2.2: PCR primer sequences for N-trm, middle and C-trm β-III spectrin fragments to clone into pL7-mCherry vector introducing *Not I* digestion site (underlined).

<table>
<thead>
<tr>
<th>Spectrin fragment</th>
<th>Forward (5'-&gt;3’)</th>
<th>Reverse (5'-&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-trm - SR4</td>
<td>ATTTGCGGCCGCCCATGAGCAGCACCCTGTCACCC</td>
<td>TAAAGCGGCCGCTACTGTGCTAGCCGCTGGGCTCG</td>
</tr>
<tr>
<td>SR2 - SR15</td>
<td>ATTTGCGGCCGCCCATGCAACTGGCTGCTCGCTTCGAC</td>
<td>TAAAGCGGCCGCTAATCCAGTAACAGCTGGCGACG</td>
</tr>
<tr>
<td>SR13 - C-trm</td>
<td>ATTTGCGGCCGCCCATGCTTCGAGCCCAGCAATTCTATCGT</td>
<td>TAAAGCGGCCGCTACTTGTCTTTCTTTAAAAGAAGCTGAATCG</td>
</tr>
<tr>
<td>N-trm - C-trm</td>
<td>ATTTGCGGCCGCCCATGAGCAGCACCCTGTCACCC</td>
<td>TAAAGCGGCCGCTACTTGTCTTTCTTTAAAAGAAGCTGAATCG</td>
</tr>
</tbody>
</table>
Transfected HEK293FT cells were placed on ice, the medium was replaced by homogenization buffer (20 mM HEPES (Sigma) pH 7.4, 2 mM EDTA (Sigma), 1× Protease inhibitor cocktail III (Calbiochem), 1 mM phenylmethanesulfonyl fluoride (Sigma)). The cells were scraped off and spun down at 425×g for 2 min at 4 °C. The supernatant was removed and the pellet was re-suspended in fresh homogenization buffer. 8-20 µg protein was mixed with the same volume of 2× loading buffer pyronin Y (125 mM Tris (Melford) pH 6.8, 20% (v/v) glycerol (BDH), 4% (v/v) sodium dodecyl sulphate (SDS, Melford), 10% (v/v) beta-mercaptoethanol (Sigma) and a pinch of pyronin Y dye (Sigma)), and boiled at 100 °C for 10 min. The samples were run on 7.5% resolving acrylamide gel (375 mM Tris-HCl (Sigma) pH 8.8, 0.1% (w/v) SDS (Melford), 7.5% (w/v) Acrylamide/Bis Solution (Sigma-Aldrich), 0.1% (w/v) ammonium persulphate (APS) (Bio-Rad), 0.4% (w/v) TEMED (Bio-Rad)) with stacking gel on top (125 mM Tris-HCl (Sigma) pH 6.8, 3.9% (w/v) Acrylamide/Bis Solution (Sigma-Aldrich), 0.1% (w/v) SDS (Melford), 0.1% (w/v) APS (Bio-Rad) and 0.4% (w/v) TEMED (Bio-Rad)) at 200 V for 40 min in a running buffer (25 mM Tris-HCl (Sigma), 52 mM glycine (Fisher Scientific), 0.1% (w/v) SDS (Melford)). The proteins were transferred to nitrocellulose membrane (Amersham) for 1 hr at 100 V in transfer buffer (25 mM Tris-HCl (Sigma), 192 mM glycine (Fisher Scientific), 20% (v/v) methanol (Fisher Chemical)). The membrane was then blocked in blocking solution (BS, 5% (w/v) milk (Marvel) with 1× Tris-buffered saline (TBS, 80 mM Tris (Melford), 419 mM Tris-HCl (Sigma), 2 M NaCl (Fisher Scientific) in dH2O), 0.1% (v/v) Tween (Sigma)) for 1 hr at RT. The primary antibody (mouse anti-c-myc (1:400, Calbiochem, catalog number OP10), rabbit anti-DsRed (1:1000, Clontech, catalog number 632496), mouse anti-actin (1:1600, Sigma, catalog number A4700)) was applied in BS overnight at 4 °C with shaking. After washing 3× 20 min in TBS-Tween, the appropriate secondary antibody (HRP-conjugated donkey anti-rabbit IgG (Amersham Pharmacia), Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Amersham)) was added at 1:3000 dilution in fresh BS and incubated at RT for 1 hr with shaking. Secondary antibody was washed.
off 5× 5 min in TBS-Tween and after getting rid of excess fluid, A and B solution (Amersham, ECL Prime Western Blotting Detection Reagent) mixture was applied for enhanced chemiluminescence for 1 min and the proteins visualised on radiographic film. The expected protein weights were calculated by adding fragment weight to either 1.2 kDa of myc tag weight for myc-tagged proteins or 30 kDa mCherry tag weight for mCherry-tagged fragments. Homogenates prepared from non-transfected HEK293FT cells were run alongside transfected HEK293FT cell homogenates to identify exogenous proteins.

2.3 Dissociated primary cerebellar cultures

All animal experiments were carried out according to the guidelines laid down by the University of Edinburgh’s Animal Welfare Committee and in accordance with the UK Animals (Scientific Procedures) Act 1986. Dissociated postnatal cultures were performed adapting previously published methods (Tabata et al., 2000; Wagner et al., 2011). Prior to culturing, cover slips were rinsed twice with dH2O and 50 µg/ml poly-L-lysine (Sigma-Aldrich) was applied for at least 1 hr at 37 °C. Mice pups of P0/P1 were anesthetized with isoflurane (Zoetis) and decapitated. The heads were put into ice cold supplemented Hank’s Balanced Salt Solution (HBSS, Thermo Fisher Scientific) with 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma)). Using forceps and scissors the brain was removed into supplemented HBSS dish under the dissection microscope. The meninges were then peeled away and the cerebellum primordial was cut off. The dissected cerebella were kept on ice in supplemented HBSS. After finishing dissections, the supplemented HBSS was replaced with 1 ml papain (10 U/ml) solution (Worthington Biochemical Corp) and incubated for 30 min at 37 °C agitating gently every 10 min. Following incubation the cerebella were washed twice in pre-warmed supplemented HBSS and triturated with a polished glass pipette for 5 min in 0.05% (w/v) DNase (Roche). Following trituration the suspension was spun down for 5 min at 104× g. The supernatant was removed and the pellet was re-suspended in 100 µl/cerebellum supplemented Purkinje cell media (Dulbecco’s Modified Eagle Medium:Nutrient Mix-
ture F-12 (Thermo Fisher Scientific) with 100 µM putrescine dihydrochloride (Sigma), 30 nM sodium selenite (Sigma), 1.4 mM L-glutamine (Sigma), 40 nM progesterone (Sigma), 20µg/ml bovine insulin (Sigma), 200 µg/ml transferrin (Sigma), 0.5 ng/ml 3,3’,5-triiodo-L-thyronine sodium salt (Sigma) and 100 µg/ml bovine serum albumin (Sigma)) with 10% (v/v) hFBS (Gibco). 80 µl of cell suspension was plated on each cover slip, 3 cover slips in a 6-well plate, which had been washed twice with dH$_2$O and air dried prior to plating. Cells were allowed to settle for 3 hr at 37 °C before 2 ml Purkinje cell supplemented medium with 10% (v/v) hFBS (Gibco) was added to each well in a 6-well plate. Cells were fed every 7 days by replacing half of the medium with fresh Purkinje cell supplemented medium and cytosine arabinoside (AraC, Sigma) was added at a final 4 µM concentration. For co-cultures of wild type (WT) and β-III spectrin knock-out (KO) mice, various ratios of WT to KO pups were used and the pups were mixed prior to the culturing process. For analysis purposes, WT to KO pup ratios of 1:5 and 1:7 were grouped as 1:7, as no significant differences were detected between the grouped ratios.

2.3.1 Drug application

For chronic drug applications mibefradil (Tocris, dissolved in water) was applied at 0.5 µM, 2 µM and 4 µM at the day of culturing while KN62 (Merck Millipore, dissolved in DMSO) was applied at 2.5 µM, 5 µM and 10 µM concentrations. During feeding 7 days later, it was assumed that all drug would be inactivated, therefore a new full dose was added. For temporary applications of mibefradil and KN62, the drugs were applied at 7 DIV.

2.4 Primary hippocampal culture and nucleofection

Prior to culturing, cover slips were washed twice with dH$_2$O and 50 µg/ml poly-L-lysine (Sigma-Aldrich) was applied for 48 hr at 4 °C. 4 hr before plating cells, the poly-L-lysine was removed and replaced with 10 µg/ml fibronectin (Life Technologies) at RT. A pregnant rat with embryos E17-E18 was euthanized with cervical disloca-
tion. The embryos were removed into ice cold Basal Medium Eagle (BME, Gibco) and allowed to cool. Each embryo was decapitated, the skin and skull peeled away and the brain scooped out into Hank’s/Hepes Solutions (HHS, 10 mM HEPES (Sigma) in HBSS (Gibco), pH 7.3). Each hippocampus was dissected out and stored in ice cold HSS supplemented with 50 µg/ml DNAse (Sigma) and 180 U/ml trypsin (Worthington Biochemical Corp). After all hippocampi were collected, they were transferred to 37 °C for 30 min. After incubation they were washed twice with BME and 10% (v/v) HHS mixture and triturated with a fire polished pipette for 5 min. Cell concentration was counted with haemocytometer and the suspension was spun down at 128× g for 3 min. The cell pellet was then re-suspended in 100 µl RT nucleofector solution (Nucleofector Kits for Rat Neurons, Lonza). The suspension was mixed with 4.5 µg of the desired DNA and 0.5 µg pMAX-GFP supplied with the kit. The programme used was O-003 (Rat Hippocampal Neurons). The cells were immediately transferred into supplemented hippocampal medium (50 U/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), 0.5% (w/v) glucose (Sigma), 1 mM sodium pyruvate (Gibco), 1× N2 (Gibco) and B27 (Gibco)) with 10% (v/v) horse serum (HS, Gibco) and plated on cover slips. Cells were plated at 700,000-1,000,000/cover slip density. The next day medium was replaced by serum-free supplemented medium and the cells were fed every 3-4 days replacing half of the well volume with fresh serum-free supplemented medium. Electrophysiological recordings were performed at 7-8 DIV.

2.5 Immunofluorescence

2.5.1 Cells on cover slips

24 hr after transfection of HEK293FT cells or following 12-14 DIV for dissociated cerebellar cultures, the cells were fixed for 20 min with 4% (w/v) PFA (Sigma) solution and then washed twice with 0.1 M Sorensen’s phosphate buffer (81:19 ratio of 0.2 M HNa₂PO₄·2H₂O (pH 7.4, Sigma) and 0.2 M H₂NaPO₄·2H₂O (pH 7.4, Sigma) diluted 1:1 in dH₂O). The cells were then permeabilized with 0.4% (v/v) triton X-100
(Sigma) in Phosphate Buffered Saline (PBS, Sigma) for 15 min and then blocked for 1 hr in 5% (v/v) normal goat serum (NGS, Jackson Immuno Research) or HS (Gibco) in PBS together with 0.1% (v/v) triton X-100. Primary antibody (mouse anti-c-myc (Calbiochem catalog number OP10), rabbit anti-C-trm β-III spectrin (generous gift from Jeffrey Rothstein), rabbit anti-DsRed (Clontech, catalog number 632496), mouse anti-calbindin (Swant, catalog number 300), rabbit anti-IP3 receptor (anti-ITPR) (Millipore, catalog number AB5882)) was added at 1:50 dilution in PBS for 1 hr together with 2% (v/v) NGS/HS and 0.1% (v/v) triton X-100. The secondary antibody (Fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Cappel), DyLight 488 conjugated goat anti-mouse IgG (Thermo Scientific), cyanine 3 (Cy3) conjugated goat anti-mouse IgG (Jackson Laboratories), Cy3 conjugated donkey anti-rabbit IgG (Jackson Laboratories)) was applied at 1:200 dilution in PBS for 1 hr. The cover slips were washed in PBS 3× 5 min after incubation in primary and secondary antibodies. The cover slips were briefly washed in dH2O before mounting them in hard- or soft-set Vectashield mounting medium with or without DAPI (Vectorlabs). To confirm antibody specificity non-transfected HEK293FT cells were immunostained with primary antibody alone as well as primary antibody together with secondary antibody and transfected HEK293FT cells were immunostained with secondary antibody only.

2.5.2 Cerebellar slices

Brains were removed and immersion-fixed with 4% (w/v) paraformaldehyde (PFA, Sigma) in 0.1 M Sorensen’s phosphate buffer (pH 7.4) overnight at 4 °C and cryoprotected by immersion in 0.1 M sodium phosphate buffer (pH 7.4) containing 30% (w/v) sucrose (Sigma). The tissue was quick-frozen on dry-ice and kept at -80 °C until 30 µm-thick coronal slices were cut with cryostat or 50 µm thick sagittal slices were cut with freezing microtome. Alternatively, 80 µm thick sagittal slices were cut during fresh-brain slicing procedure preparing for slice electrophysiology and immersed in 4% (w/v) PFA overnight. Free-floating cerebellar sections were then permeabilised by washing slices 3× 5 min in PBS with 0.5% (v/v) triton X-100. The sections were then blocked
for 3 hr in PBS with 5% (v/v) NGS (Jackson Immuno Research) and 0.1% triton X-100 (v/v). After washing them 3× 5 min in PBS, primary antibody (mouse anti-calbindin (Swant, catalog number 300) or rabbit anti-ITPR (Millipore, catalog number AB5882)) was applied at 1:50 dilution with 2% (v/v) NGS (Jackson Immuno Research) and 0.1% (v/v) triton X-100 (Sigma) at 4 °C overnight with gentle shaking. 3 × 5 min washes were performed in PBS the next day and the secondary antibody (Cy3 conjugated goat anti-mouse IgG (Jackson Laboratories) or Cy3 conjugated donkey anti-rabbit IgG (Jackson Laboratories)) was applied at 1:200 dilution in PBS for 2 hr. After 3 × 5 min wash in PBS and rinse in dH$_2$O, the slices were mounted onto poly-L-lysine (Sigma-Aldrich) coated microscope slides with hard-set vectashield (Vectorlabs). Brain slices with viral particles were mounted onto microscope slides directly after fixing and slicing, without any staining steps performed. As a control, slices were immunostained with either primary or secondary antibody alone.

### 2.6 Electrophysiology

#### 2.6.1 Hippocampal pyramidal neurone culture

Whole-cell voltage clamp recordings were performed at 7-8 DIV in external solution containing the following (in mM): 130 NaCl (Sigma), 20 TEA-Cl (Sigma), 3 KCl (Sigma), 1 MgCl$_2$ (Sigma), 1 CaCl$_2$ (Sigma), 10 Glucose (Sigma), 10 HEPES (Sigma), 0.2 CdCl$_2$ (Aldrich), pH 7.3 with NaOH (AnaR). For recording sodium currents the internal solution contained the following (in mM): 140 CsF, 1 EGTA (Sigma-Aldrich), 10 NaCl (Sigma-Aldrich), 10 HEPES (Sigma), pH 7.3 with CsOH (Sigma). The holding potential was -90 mV which allowed sodium currents to be investigated. Voltage steps were applied at 10 mV increments from -80 mV to +20 mV with each lasting 50 ms in duration and data were filtered at 2 kHz and sampled at 10 kHz frequency. Series resistances were <15 MΩ and were compensated for by 85%. The sodium current size was measured for each voltage step and transfected cell sodium currents were normalised to non-transfected pyramidal neurones in the same culture to allow comparisons between
cultures.

2.6.2 Dissociated cerebellar cultures

Purkinje cells were visualized with 40× immersion objective and Normarski differential interference contrast optics. Whole-cell recordings were obtained from Purkinje cells using thick-walled borosilicate glass pipettes pulled to 3–6 MΩ at 10 DIV or 14-18 DIV at 30 °C. For recording action potentials and cellular response to current-injection protocol, external solution contained the following (in mM): 150 NaCl (Sigma), 2.8 KCl (Sigma), 10 HEPES (Sigma), 2 CaCl₂ (Sigma), 1 MgCl₂ (Sigma), 10 glucose (Sigma), pH 7.3 with NaOH (AnalR). Picrotoxin (PTX, Abcam) was added to the external solution at 50 µM final concentration and NBQX (Abcam) was added 1-10 µM to the external solution either before the recording or 2 min after recording had started. All results obtained with 1-10 µM NBQX were grouped together as no statistical differences were found between cell responses with different NBQX concentrations. The internal solution for recording action potentials contained the following (in mM): 130 KGluc, 10 KCl (Sigma), 10 HEPES (Sigma), 2 MgCl₂ (Sigma), 0.1 EGTA (Sigma-Aldrich), 2 NaATP (Sigma), 0.3 Mg₂GTP (Sigma), 10 Na phosphocreatine (Sigma), pH 7.4 with KOH (Sigma). After recording spontaneous cell responses, the cells were driven to fire with current injection (0-700 pA) for 1 s at 50 pA increments, data were filtered at 2 kHz and sampled at 100 kHz. Analysis of cell response was performed using custom-built MATLAB scripts which automatically sets a threshold for action-potential detection. Series resistances were <15 MΩ and were compensated for by 85%. For spontaneous firing activity, 50 s long firing stretches were analysed 50 s after breaking into the cell or 50 s after NBQX application using custom-built MATLAB Scripts. Data were filtered at 2 kHz and sampled at 10 kHz frequency. Only the cells spontaneously firing for 100 s were included in the analysis. All recordings obtained at 14-18 DIV were grouped together as no differences were observed between cell firing frequencies or input/output plots. Input resistance was calculated according to Ohm’s law (I=V/R) by measuring steady state voltage deflection after a hyperpolarising step. Coefficient
of variation (CV) was calculated as a ratio between standard deviation of interspike interval (ISI) and mean ISI value. CV values were obtained by analysing 50 s of Purkinje cell spontaneous firing activity.

2.6.3 Cerebellar slices

Cerebella were dissected out into ice-cold modified artificial cerebrospinal fluid (ACSF) containing the following (in mM): 60 NaCl (Sigma), 118 sucrose (Sigma), 26 NaHCO$_3$ (Sigma), 2.5 KCl, 11 glucose, 1.3 MgCl$_2$ (Sigma), and 1 NaH$_2$PO$_4$ (Acros Organics) at pH 7.4 when bubbled with 95% O$_2$:5% CO$_2$. The cerebellar vermis was glued to the vibratome cutting platform (Leica VT1200S) with ethyl-2-cyanoacrylate adhesive. 220 µm-thick sagittal slices were cut and incubated for 30 min at 30 °C in standard ACSF composed of the following (in mM): 119 NaCl (Sigma), 2.5 CaCl$_2$ (Sigma), 26 NaHCO$_3$ (Sigma), 2.5 KCl (Sigma), 11 glucose (Sigma), 1.3 MgCl$_2$ (Sigma), and 1 NaH$_2$PO$_4$ (Acros Organics) pH 7.4 when bubbled with 95% O$_2$:5% CO$_2$. Slices were stored at room temperature until required for recording. Slices were transferred to a submerged recording chamber and superfused with standard ACSF (3–5 ml/min) at room temperature for spontaneous firing recording and current clamp experiments at 30±1 °C for recording spontaneous action potentials. Purkinje cells were visualized with 40× immersion objective and Normarski differential interference contrast optics. Whole-cell recordings were obtained from Purkinje cells using thick-walled borosilicate glass pipettes pulled to 3–6 MΩ. For recording action potentials, the internal solution contained the following (in mM): 125 K-gluconate (Sigma), 15 KCl (Sigma), 10 HEPES (Sigma), 5 EGTA (Sigma), 2 MgCl$_2$ (Sigma), 0.4 NaGTP (Sigma), 2 NaATP (Sigma), and 10 Na-phosphocreatine (Sigma), adjusted to pH 7.4 with KOH (Sigma). PTX (50 μM, Abcam) and NBQX (10 μM, Abcam) were added to the ACSF. 50 s long spontaneous firing stretch was analysed 50 s after breaking into the cell using custom-built MATLAB Scripts by setting a threshold. Data were filtered at 2 kHz and sampled at 10 kHz frequency. Only the cells spontaneously firing for 400 s were included in the analysis. After recording spontaneous cell responses, the cells were driven to fire...
with current injection (0-700 pA) for 1 s at 50 pA increments and sampled at 100 kHz. Analysis of cell response was performed using custom-built MATLAB scripts which automatically sets a threshold for action-potential detection. GFP expression strength was observed under the microscope and each Purkinje cell was given an arbitrary value for GFP expression ranging from 1 (hardly visible) to 5 (very bright). For cell filling experiments, individual Purkinje cells were filled with 0.02 mM Alexa Fluor 568 Hydrazide (Invitrogen) (Gao et al., 2011). The dye was included in patch pipette and whole-cell patch-clamp recording was made. Cells were voltage clamped at -60 mV and given a 20 ms -10 mV step at 50 Hz for up to 30 min. Level of dye filling was monitored intermittently to prevent bleaching. After removal of patch electrode the slice was immediately removed into 4% PFA and left at 4 °C overnight. Slices were then washed in PBS followed by rinse in water and stored in soft-set vectashield (Vectorlabs) until imaged. Dr Emma Perkins assisted with the filling of some Purkinje cells. Anterior cerebellum in electrophysiological experiments was considered as Purkinje cells from lobules II to IV and posterior cerebellum was regarded as lobules VIII-X. Input resistance was calculated according to Ohm’s law (I=V/R) by measuring steady state voltage deflection after a hyperpolarising step. CV was calculated as a ratio between standard deviation of interspike interval (ISI) and mean ISI value. CV values were obtained by analysing 300 s of Purkinje cell spontaneous firing activity.

2.6.4 Recording set up

All of the recordings were made using MultiClamp 700B Amplifier, data were digitized with a Axon Digidata 1550A interface with pClamp 10 software (Axon Instruments, Foster City, CA, USA) and analysed using Clampfit 10.

2.7 Viral particle production

Viral particles were generated as described previously (McClure et al., 2011). Approximately 24 hr prior to DNA transfection confluent HEK293FT cells were plated from 175 cm² flask onto three 15 cm dishes in supplemented HEK cell medium (Section 2.2)
Chapter 2 Methods

until ∼70% confluency was reached for calcium phosphate transfection. 3 hr prior to the transfection supplemented medium was replaced with pre-warmed Iscove’s Modified Dulbecco’s Medium (IMDM, Life Technologies) supplemented with 5% (v/v) FCS (Life Technologies). Transfection mixture consisted of 2.4 ml dH$_2$O, 318 mM CaCl$_2$ (Sigma), 12.5 µg previously prepared pAAV-Gateway backbone with C-trm β III-spectrin (produced using Gateway Cloning technology from Thermo Fisher Scientific) with the following helper plasmids: 25 µg pFΔ6, 6.25 µg pRV1 and 6.25 µg pH21. The mixture was then filtered through 0.2 µm filter (Iso-Disc with PVDF membrane, Sigma) and an equal volume of HEBS was added while vortexing vigorously for 30 s and the mixture was let to settle for another 1 min 45 s. The transfection mixture was then added to the plate in a dropwise circular motion and swirled gently to mix. 16 hr after the transfection the medium was replaced with 25 ml of fresh pre-warmed Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies) with 10% (v/v) FCS (Life Technologies), 1% (v/v) non-essential amino acids (Life Technologies), 1% (v/v) sodium pyruvate (Life Technologies) and 0.5% (v/v) Penicillin/Streptomycin (Life Technologies). Viral particles were harvested 60-65 hr after transfection after washing the cells with pre-warmed PBS and scraping them off in 10 ml PBS followed by centrifugation at 800× g for 10 min. Pellet was resuspended in 150 mM NaCl (Sigma), 20 mM Tris (Melford) solution at pH 8.0. Sodium deoxycholate (Sigma) and benzonase endonuclease (Sigma) were added at final concentrations of 0.5% (w/v) and 50 U/ml, respectively. The mixture was then incubated at 37 °C for 1 hr. Cell debris was removed after centrifugation for 15 min at 3000× g and supernatant passed through heparin column (1ml HiTrap Heparin columns, Sigma) which was pre-equilibrated with 10 ml 150 mM NaCl (Sigma), 20 mM Tris (Melford) solution with pH 8.0 at 1 ml/min flow rate. Column was washed with 20 ml 100 mM NaCl (Sigma) and 20 mM Tris (Melford) pH 8.0 followed by 1 ml 200 mM NaCl (Sigma), 20 mM Tris (Melford) pH 8.0 and 1 ml 300 mM (Sigma) NaCl (Sigma), 20 mM Tris (Melford) pH 8.0. The eluent after applying 1× 1.5 ml 400 mM NaCl (Sigma), 20 mM Tris (Melford) pH 8.0, 3 ml 450 mM NaCl (Sigma), 20mM Tris (Melford) pH 8.0 and 1.5 ml 500 mM NaCl (Sigma), 20 mM Tris (Melford) pH 8.0 was
collected in a sterile tube and concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore) to around 250 µl. Viral particles were filtered through 0.2 µm filter, aliquoted and stored at -80 °C. Establishing the functional viral titre measuring the GFP fluorescence after serial dilution on HEK293FT cells was unsuccessful. Virus production was performed 3 times in order to increase the viral titre. The second batch of virus, with higher titre was produced by concentrating viral particles to 100 µl instead of 250 µl. The third batch of virus was produced by transfecting double the amount of HEK293FT cells and concentrating it to 100 µl.

2.8 Image capture and analysis

2.8.1 Microscopy

Confocal images were captured with either a Zeiss inverted LSM510 or Nikon confocal laser scanning microscope at the University of Edinburgh IMPACT imaging facility. Co-localisation analysis was carried out using FIJI (Fiji Is Just ImageJ, 1.6.0-24) Coloc-2 plugin while the percentage of YFP plasmid expressing cells was calculated by visualizing all cells with DAPI staining. Cerebellar slices with filled cells were imaged as described previously (Gao et al., 2011).

In dissociated cerebellar cultures cell surface area was quantified with in-house built MATLAB script. The number of cell dendritic branches and total dendritic length was obtained running Scholl analysis in Neuronstudio software. Branch density value was obtained by dividing the number of branching points by total dendritic length and multiplying by 100.

Quantification of viral transduction efficiency after the first viral batch injection was carried out blind to genotype by counting Purkinje cell bodies expressing GFP under at 20× Olympus IX70. The spread of the virus was calculated as the sum of thickness of slices expressing the virus, with each slice being 80 µm thick. Density of virally transduced cells was calculated by dividing the total number of GFP expressing cells by the total spread and multiplying by 100.
Dendritic tree morphological analysis in cerebellar slices after viral injection or cell-filling, was performed by calculating the surface area covered by the dendritic tree (dendritic field) of Purkinje cell and it was achieved by connecting the outermost dendritic tips in the FIJI software using the polygon selection tool as described previously (Pascual et al., 2014; Yoshimatsu et al., 2016). Distance to the the first dendrite was measured as a distance between cell body and first dendrite on a maximum intensity z-projection in FIJI. The distance to the dendritic tree was measured from cell body to the first dendrite, which in Z projection overlaps with other dendrites. Surface area of filled Purkinje cells with good signal to noise ratio was also evaluated by using in-house built MATLAB script.

All quantification in cell density analysis was carried out blind to genotype and involved counting the number of Purkinje cells in the anterior (lobules I-V, simplex and crus I) and posterior cerebellum (lobules VI -IX, crus II and flocculonodular lobe, lobule X) from three sections/animal and the counts averaged and was carried out by Cyra Lee.

### 2.9 Stereotaxic viral injection

β-III spectrin KO and WT mice aged 6-12 weeks were anaesthetized with isoflurane (Zoetis) and mounted in a stereotaxic frame (David Kopf Instruments). The virus was delivered as previously described (White et al., 2011) via pre-callibrated pulled glass pipettes with the total injection of 0.6-3 µl of virus at 100 nl/min rate. Injections were measured relative to lambda: -2 A/P, 0 M/L either at Z= -3, -2.5, -2, -1.5, -1 with injection arm angled 10°back or Z=-4, -3.5, -3, -2.5, -2 with injection arm at 25°angle and Z=-2,-1.5,-1 and -0.5 with 10° injection angle. 100–300 nl were injected at each depth and the glass pipette was left in place for 5 min at each step. After the surgery animals were returned to their cages for at least 2 weeks before commencing experiments and their well being was evaluated for at least 5 days after the surgery.


**2.10 Mouse lines**

The main mouse line used for the outlined experiments was a previously published β-III spectrin KO mice (Perkins et al., 2010). Other mouse lines also reported in this thesis had GFP expression under Excitatory Amino Acid Transporter 4 (EAAT4) promoter with and without alterations in β-III spectrin gene, allowing straightforward visualisation of Purkinje cells under the microscope and were used as WT and KO animals, respectively.

**2.11 Animal behaviour**

The rotarod, elevated beam and footprint analysis were performed prior to the surgery and repeated 2 weeks after with monthly repetitions thereon for long term expression experiments until the animals were sacrificed for histological and/or electrophysiological analysis. Each animal experimental group was handled by the experimenter for at least a week prior to the start of the experiment. The animals were also allowed to get used to the test room.

**2.11.1 Rotarod**

In the rotarod test, the ability of mice to maintain balance on a rotating (3, 5 and 10 rpm) 3 cm diameter cylinder was assessed (TSE Rotarod) by measuring the time a mouse remained on the moving cylinder (maximum, 120 s). The experiment was performed for 4-5 consecutive days, with 4 trials recorded daily. Animals were allowed to rest between the trials for 5-30 min.

**2.11.2 Elevated beam**

The elevated beam test was performed using a narrow horizontal beam (2 cm wide, 80 cm long, held at a height of 30 cm from the table). The time taken to cross the beam as well as the number of hind-paw slips the animal made while traversing the beam was recorded.
2.11.3 Footprint pattern analysis

Footprint patterns were analyzed using a runway (80 cm by 10.5 cm wide) with white paper at the bottom. Hind paws of animals were dipped in nontoxic, water-soluble black ink (Indian Ink, Winsor & Newton). Three consecutive strides were measured for each animal. Stride length measurements were taken from the base of two consecutive paw prints on the same side, and the base width was measured as the distance between the centre of one paw print to the centre of the next print on the opposite side.

2.12 Statistical analysis

Statistical analysis was performed using Student’s t-test for 2 groups of data and one-way analysis of variance (ANOVA) for comparison of 3 or more data groups. Two-way analysis of variance (two-way ANOVA) was used for comparison of 2 or more data sets over a range of voltage, current or time. Contingency table and Fisher’s tests were used for categorical data evaluation. Contingency table investigates the effect of treatment (such as drug application) or different experimental settings when there are only two categorical outcomes (for example, the cells fire or remain silent) (Motulsky). Linear regression test was performed and $r^2$ value is reported for evaluating Purkinje cell transduction by viral particles. All data is plotted as mean $\pm$ standard error of the mean (SEM). Indication of significance is as follows: $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. N indicates the number of animals or cultures and is reported above or below data points in figures or in figure legends, while n indicates the number of individual cells analysed for each animal or cell culture.
Chapter 3

Purkinje cell heterogeneity across different cerebellar regions

3.1 Introduction

Ever since the discovery of Purkinje cells in 1837 by Jan Evangelista Purkinje (Purkinje, 1838) and a detailed description of the dendritic tree made by Camillo Golgi (Golgi and Fischer, 1894), the cerebellum has been thought of as a homogenous structure. Indeed, all of the cerebellar cortex, regardless where in the cerebellum, consists of the same cerebellar circuitry repeats with Purkinje cells receiving excitatory input from both climbing fibres and granule cells and in turn being the sole output of the cerebellum (Ramón y Cajal, 1904). However, recently it has been shown that the cerebellum is more heterogenous than previously thought. Several proteins, such as zebrin II (Brochu et al., 1990), Excitatory Amino Acid Transporter 4 (EAAT4) (Dehnes et al., 1998) and phospholipase Cβ4 (PLCβ4) (Sarna et al., 2006) have been shown to be expressed in a stripe-like fashion with groups of Purkinje cells expressing the protein in question at high or low level.

Purkinje cells have been demonstrated to be spontaneously active during electrophysiological recordings (Woodward et al., 1974) even in the absence of synaptic input (Häsüser et al., 1997). As Purkinje cells provide the sole output from the cerebellar
Chapter 3 Purkinje cell heterogeneity across different cerebellar regions

cortex their firing mode and frequency is therefore a useful tool in evaluating cerebellar function in murine models of health and disease. However, different cerebellar regions have also been demonstrated to have distinct electrophysiological properties. A study by Zhou et al. (2014) showed that zebrin negative Purkinje cells fired simple spikes at significantly higher frequencies than their neighbouring zebrin positive Purkinje cells. This was confirmed by a study by Xiao et al. (2014) in which authors showed that these differences between firing frequencies of zebrin positive and negative Purkinje cells persist in various cerebellar regions.

The posterior cerebellum was also shown to have lower Purkinje cell firing rates when compared to anterior cerebellar regions (Kim et al., 2012; Witter and De Zeeuw, 2015). As zebrin negative Purkinje cells are mostly found in the anterior cerebellar regions, some experimenters have suggested that the cerebellar localisation of Purkinje cells rather than the level of zebrin expression has more effect on their firing properties (Zhou et al., 2014). Passive membrane properties of Purkinje cell membranes have also been demonstrated to differ with anterior cerebellum showing significantly higher input resistance values when compared to posterior cerebellum suggesting that Purkinje cells from posterior cerebellum are less excitable than the ones from anterior cerebellum (Kim et al., 2012). Origins of cerebellar inputs also differ: anterior regions receive input from sensory brainstem nuclei, lobules V to IX get input from pontine nuclei while lobule X receives vestibular input (Witter and De Zeeuw, 2015).

It has been suggested that during normal ageing neuronal numbers reduce in some brain regions such as cerebellum and hippocampus (Rogers et al., 1984; Woodruff-Pak, 2006; Woodruff-Pak et al., 2010; Zhang et al., 2011). In the cerebellum, for example, lobule VII was reported to lose significantly more Purkinje cells during the normal ageing process (Dlugos and Pentney, 1994). Similarly, studies by Koller et al. (1981) and Luft et al. (1999) reported cerebellar vermis being significantly more affected by age-related degeneration than cerebellar hemispheres. However, the data in this area are conflicting, with some researchers observing no cell loss in healthily ageing individuals (Scheibel, 1979; Merrill et al., 2001).
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Additionally, in some brain regions morphological neuronal changes have been reported to occur: dendritic length is observed to reduce in the Wernicke’s area of healthy older human subjects (Jacobs and Scheibel, 1993). In the cerebellum, not only has Purkinje cell death been observed, but also remodeling of the dendritic tree. For instance, in cats and rats thinning of the molecular layer is accompanied by reduction in the dendritic area (Rogers et al., 1984; Dlugos and Pentney, 1994; Hadj-Sahraoui et al., 2001; Zhang et al., 2011; Kennard et al., 2013), decrease of dendritic branches is observed in rats (Pentney, 1986) as well as reduction in the number of synapses (Glick and Bondareff, 1979) and dendritic spines (Zhang et al., 2011). These morphological changes have been shown to correlate with functional abnormalities such as slower firing rates in ageing rats (Rogers et al., 1980) and impaired learning of classical eye-blink conditioning (Woodruff-Pak, 2006; Woodruff-Pak et al., 2010). However, other researchers have demonstrated that dendritic length is not affected during ageing (Flood et al., 1985, 1987; Hanks and Flood, 1991) and some did not find cell loss in the cerebellum (Druge et al., 1986; Bakalian et al., 1991).

In this chapter the evaluation of heterogeneity in cellular, morphological and electrophysiological properties of Purkinje cells within the anterior and posterior cerebellum of the β-III spectrin knock-out (KO) mice will be presented.
3.2 Results

3.2.1 Purkinje cell density reduction in posterior cerebellum

In order to investigate the progression of neurodegeneration, anterior and posterior coronal cerebellar slices were first stained with anti-calbindin antibody for Purkinje cell detection (assisted by Cyra Lee) as seen in Fig. 3.1a. Image quantification in Fig. 3.1b reveals that the cell density in the posterior cerebellum of KO mice is only 55.5±7.8% of wild type (WT) animal values.

3.2.2 Dendritic tree changes in ageing mice

To further analyse the progression of neurodegeneration, individual Purkinje cells from various cerebellar regions were filled with Alexa Fluor 568 Hydrazide in sagittal cerebellar slices of WT and KO mice at various ages (Fig. 3.2). Quantification of dendritic tree morphology using FIJI software by connecting the outermost points of dendritic tree (polygon selection tool) has revealed that in WT and KO mice (Fig. 3.3a and b, respectively) Purkinje cells in the posterior cerebellum get significantly smaller with age with slight increase in the surface area at 18 months of age. This result, however, could be due to low number of filled cells obtained for 18 months data point. The anterior regions, however, did not undergo any significant changes. Moreover, in KO mice Purkinje cells from posterior cerebellar regions were significantly smaller when compared to anterior regions (Fig. 3.3b), while no differences were observed in WT mice (Fig. 3.3a). For 6 week time point, few animals were investigated (1 for WT, 2 for KO) for Fig. 3.3 due to tissue availability.

When the genotypes were compared, Purkinje cells from anterior lobules of WT mice were found to have significantly larger dendritic trees at most investigated time points with 18 months exception when compared to KO mice (Fig. 3.3c), while in posterior cerebellum all the developmental stages showed significantly bigger dendritic trees in WT mice (Fig. 3.3d).
Figure 3.1: Significant Purkinje cell loss in the posterior cerebellum. a) Coronal cerebellar sections from 12 month old WT and β-III spectrin KO mice immunostained with anti-calbindin comparing anterior (Ant, lobules I-V, simplex and crus I, top panel) and posterior (Post, lobules VI-IX, crus II and flocculonodular lobe, lobule X, bottom panel). Scale bar, 200 µm b) Quantification of cell density as a percentage of WT cell density reveals significant reduction of Purkinje cells in the posterior cerebellum of KO animals when compared to WT mice (p=0.0140, Two-way ANOVA, N=3).
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(a)

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(b)
Figure 3.2: Individually filled Purkinje cells from anterior (Ant) and posterior (Post) cerebellum in WT (a) and KO (b) mice at 6 weeks, 6 months, 12 months and 18 months of age (rows, top to bottom). Scale bar, 50 µm. a) Purkinje cells in WT mice show no obvious dendritic tree changes with ageing. b) Purkinje cells in posterior cerebellum of KO mice have much smaller dendritic trees when compared to anterior cerebellum regions and exhibit dendritic tree reduction with ageing.
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Figure 3.3: Purkinje cells undergo dendritic tree changes in WT and KO mice during adulthood. a) Purkinje cells from the posterior regions of the cerebellum undergo significant dendritic tree area reduction in ageing WT mice (*p=0.0314, **p=0.0155, Two-way ANOVA, N=1-5, n=2-11) b) Only posterior, not anterior region of the cerebellum undergo significant dendritic tree area reduction in KO mice, with Purkinje cells from the posterior cerebellum showing significantly smaller dendritic trees when compared to anterior regions (***p=0.0003, ****p< 0.0001, *p=0.0262, ***p=0.0014, Two-way ANOVA, N=1-6, n=3-11) c), d) Dendritic tree surface area analysis in anterior (c, **p=0.0044, ***p=0.0005, ***p=0.0010, Two-way ANOVA) and posterior (d, *p=0.0281, *p=0.0251, **p=0.0052, *p=0.0210, Two-way ANOVA) cerebellum using FIJI software reveals smaller dendritic trees in KO mice in both areas (N=1-6, n=2-11). The numbers on the graphs indicate N.

Further analysis of Purkinje cell morphology in Fig. 3.4 reveals that Purkinje cells from the posterior cerebellum in WT (Fig. 3.4a) and KO (Fig. 3.4b) mice had a trend of increased distances to the first dendrite, however, this is only significant in KO animals at 12 months of age. Additionally, only KO mice demonstrated significant distance increase with ageing (Fig. 3.4b). Similarly, Purkinje cells from posterior cerebellum

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had a trend of longer distance between the soma and the dendritic tree in KO mice (Fig. 3.4d). This difference, however, was only significant at 18 months of age, suggesting a progressive loss of proximal dendrites with age. No such trends were seen in Purkinje cells of WT mice (Fig. 3.4c).

**Figure 3.4:** Purkinje cells in KO mice demonstrate progressive loss of proximal dendrites. a), b) Distance to the primary dendrite in WT (a) and KO (b, ****p< 0.0001, Two-way ANOVA, N=1-5, n=2-11) mice show that in Purkinje cells from the posterior cerebellum of KO mice the distance to proximal dendrite increases significantly at 12 months of age c), d) Distance to the dendritic tree in WT (c) and KO (d, *p=0.0356, Two-way ANOVA, N=1-5, n=2-11) mice show that the distance between soma and the dendritic tree significantly increases in posterior cerebellum of KO mice at 18 months of age.
3.2.3 Tonic activity

Previous publication by Perkins et al. (2010) evaluated tonic activity of Purkinje cells at 6-weeks and 6-months of age with no separation of region and found that Purkinje cells from KO mice were firing at significantly lower frequency. Therefore a study was carried out to evaluate the firing profile at 12 months of age by performing whole-cell patch clamp recordings. The aim of the experiment was to correlate neurodegenerative morphological observations with potential functional effects, which would underlie the deteriorating motor performance. Initial quantification of firing modes observed in Purkinje cells from WT animals revealed that in the anterior regions only 54.5% of the cells exhibit spontaneous tonic firing or bursting mode with 45.5% falling silent (Fig. 3.5d), while 91.7% of Purkinje cells were spontaneously firing or bursting in the posterior regions with only 8.3% of the cells falling silent. These differences were found to be statistically significant (p=0.0023) in WT mice (Fig. 3.5d). No differences between firing modes were observed in the Purkinje cells of KO mice (Fig. 3.5e), with the majority of Purkinje cells tonically firing in both, anterior and posterior areas.

When firing frequency of the spontaneously active Purkinje cells was evaluated in Fig. 3.6, it was observed that although KO Purkinje cells had lower firing frequency in both, anterior and posterior regions (24.10 and 20.49 Hz, respectively) when compared to WT animals (32.61 and 28.13 Hz), this reduction was only observed to be significant in the posterior regions of the cerebellum. In line with these findings, interspike interval (ISI) was found to be significantly longer in the posterior cerebellum (0.041 sec and 0.055 sec for WT and KO, respectively, data not shown). No difference in coefficient of variation (CV) of ISI was observed between WT and KO animals (Fig. 3.6d). However, in KO animals Purkinje cells in the posterior cerebellar regions had significantly smaller CV values.

To further investigate the electrophysiological properties of Purkinje cells, depolarising currents were injected to elicit spiking (sample traces in Fig. 3.7). It was observed that all cells recorded from had similar input resistance with a trend of higher input resistance in the posterior regions of KO mice (Fig. 3.8a). The current injection protocol
Figure 3.5: Firing modes of Purkinje cells in anterior and posterior cerebellum. a), b) and c) Sample traces of spontaneously tonically firing (a), bursting (b) and silent (c) Purkinje cell current clamp recordings, respectively, from anterior cerebellum of WT mice d) Ratio of cells in tonic firing or bursting mode and cells, which are falling silent, is significantly different in anterior and posterior cerebellar regions of WT mice (**p=0.0023, Contingency table, N=3-5, n=58) e) No differences in firing modes were observed in KO mice comparing anterior and posterior cerebellar regions (N=6, n=61).
Figure 3.6: Purkinje cells in posterior cerebellum have significantly slower firing rates in KO mice. a), b) Sample traces of spontaneously tonically firing Purkinje cells from the posterior cerebellum of WT (a) and KO (b) mice c) Analysis of spontaneous firing activity shows significantly decreased firing frequency in the posterior cerebellum of KO mice (*p=0.0328, Two-way ANOVA) d) Coefficient of variation of ISI analysis reveals significant difference between anterior and posterior cerebellum in KO mice (*p=0.0193, Two-way ANOVA, N=3-6, n=11-29).

(Fig. 3.7a) also did not reveal any significant differences in anterior cerebellar regions (Fig. 3.8b), however, in the posterior cerebellum, Purkinje cells from the KO animals
were firing at higher frequency indicating increased cell excitability (Fig. 3.8c).

As whole-cell patch clamp recordings were performed for a total of 7 minutes, it was important to establish a reliable time point for data analysis. In order to achieve that, spontaneous firing frequency at the beginning of the recording (50 sec after start of the recording) and towards the end of the recording (6 min 30 sec after the start of the recording) were compared in the same cells. In WT mice, no significant differences were found between the different recording time points either in anterior (Fig. 3.9a) or posterior (Fig. 3.9b) region of the cerebellum. In KO animals, no change in firing frequency was observed (Fig. 3.9c and d), however, the variation of firing frequency was significantly increased at the end of the recording for Purkinje cells in anterior lobules (p=0.0452) in Fig. 3.9c.
Figure 3.7: a) Current injection protocol used to drive Purkinje cells to fire b) Sample traces of Purkinje cells firing in WT (left) and KO (right) mice in response to injected depolarizing currents following the protocol and shown at 0, 150, 450 and 700 pA injection steps (rows, top to bottom).
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(a) **PC input resistance in 12mo mice**

(b) **Input/output plot for tonic Purkinje cells in anterior cerebellum**

(c) **Input/output plot for tonic Purkinje cells in posterior cerebellum**

**Figure 3.8:** Increased excitability of Purkinje cells from posterior cerebellum of KO mice at 12 months of age. a) Input resistance was consistent across different genotypes and cerebellar regions, with a small increase in KO mice posterior cerebellum Purkinje cells (N=3-6, n=5-17) b) 50 pA current injection steps into anterior cerebellum showed no differences in cell excitability (N=3-4, n=5-11) c) Current injection into posterior cerebellum revealed significantly increased excitability in KO mice Purkinje cells when compared to WT animals at 700 pA current injection step (Two-way ANOVA, *p=0.0242, N=5-6, n=12-17).
Figure 3.9: Analysis of a later period in a whole-cell current clamp recording increases frequency variation in anterior cerebellum of KO mice. a), b) Analysis of spontaneous firing of Purkinje cells from anterior (a, N=3, n=11) and posterior cerebellum (b, N=5, n=29) from WT mice at 50 seconds (50-100) and 390s (390-400) of the recording reveals no changes in spontaneous firing frequency c) Spontaneous firing frequency showed significant variations in anterior cerebellum of KO mice (N=4, n=17) d) No changes were observed in the posterior cerebellum (N=5, n=19).
3.3 Discussion

3.3.1 Posterior cerebellum in β-III spectrin KO mice is more susceptible to degeneration

The data presented in this chapter have shown that β-III spectrin KO mice show different degeneration profiles across regions of the cerebellum. It was observed that Purkinje cell density at 12 months of age in the posterior cerebellum of the KO mice is significantly decreased, while the anterior regions are relatively unaffected (Fig. 3.1). Such varying levels of neurodegeneration have also been observed in weaver mice, which show significantly higher degree of Purkinje cell death in the posterior regions when compared to anterior regions by postnatal day 90 (P90) (Martí et al., 2016). Similarly, in nervous mouse Wassef et al. (1987) reported selective Purkinje cell degeneration in the posterior cerebellar regions. In contrast, a mouse model for Niemann-Pick disease type C (NPC) shows regional Purkinje cell degeneration in the anterior cerebellum (Higashi et al., 1993).

Furthermore, data in this chapter show that Purkinje cells in posterior regions of β-III spectrin KO mice by 12 months of age show morphological abnormalities - dendritic tree surface area is significantly decreased at almost all the time points except 18 months (Fig. 3.2 and Fig. 3.3). The loss of significance at 18 months could potentially be explained by a lesser number of filled Purkinje cells available for analysis at 18 months of age and whether the cells remaining are generally healthier neurons. Of note, using the SCA1 mouse model by Burright et al. (1995), Clark et al. (1997) also reported proximal dendrite loss in cerebellar Purkinje cells.

It has been previously observed that Purkinje cells in spinocerebellar ataxia type 5 (SCA5) mouse model fire significantly slower than WT Purkinje cells (Perkins et al., 2010). In other murine ataxia models, Purkinje cell firing abnormalities have also been reported. For example, in SCA2 using transgenic mouse model ATXN2-Q127, at 6 weeks of Purkinje cell spontaneous firing rate has been reported to be significantly reduced (Hansen et al., 2013). In contrast, a study by Shakkottai et al. (2011) reported
an increase in Purkinje cell firing frequency in SCA3 mouse model. These findings suggest that disturbed output of Purkinje cells plays a role in ataxic phenotype. The data presented in this chapter, however, reveal that it is the posterior cerebellum Purkinje cells that fire at significantly lower frequencies when compared to WT animals (Fig. 3.6c). Such trend has been previously reported in WT mice (Kim et al., 2012; Witter and De Zeeuw, 2015). However, in this study no such difference was observed in WT mice.

It was also found that anterior regions of the cerebellum in old WT animals had different firing modes with more silent Purkinje cells present (Fig. 3.5d) when compared to posterior regions. Such a difference was not observed in the old KO mice (Fig. 3.5e). The data also showed that Purkinje cells from anterior regions of KO mice seem to change the distribution of their firing frequency significantly during prolonged recordings (Fig. 3.9c) with no such changes observed in WT animals (Fig. 3.9a, b) or posterior cerebellum of the KO mice (Fig. 3.9d).

The study by Witter and De Zeeuw (2015) indicated that the posterior cerebellum usually has lower excitability. The data in this chapter show that following injection of depolarising currents posterior cerebellum of WT mice showed a similar level of excitability when compared to anterior cerebellum but was slightly lower (p=0.1996). In contrast, Purkinje cells from KO mice revealed significantly higher excitability of the Purkinje cells from the posterior cerebellum when compared to the WT animals (Fig. 3.8c). These data suggest that electrophysiological properties of Purkinje cells in posterior cerebellum of KO mice are significantly more affected in the disease process.

Purkinje cells in anterior cerebellum have been reported to have higher input resistance when compared to posterior cerebellum when recorded from P23 rats (Kim et al., 2012). The data in this chapter did not show any differences in input resistance in WT mice at 12 months of age. In contrast, Purkinje cells from posterior cerebellar regions of KO mice had elevated, but not significant, input resistance values when compared to anterior cerebellum as well as WT mice. As Purkinje cell dendritic trees are severely impaired in the posterior cerebellum, higher input resistance value could be caused by
 smaller cell size and different cellular shape of Purkinje cells in KO mice. Alternatively, changes in input resistance might be caused by fewer leak channels being expressed in KO mice posterior cerebellum Purkinje cell membranes.

The slower firing frequency in Purkinje cells from KO mice (Perkins et al., 2010) has been proposed to be caused by sodium channel dysfunction (Perkins et al., 2016a). Smaller sodium currents have been previously reported in SCA5 mouse model (Perkins et al., 2010) and it might be a result of sodium channel instability due to abnormalities in ankyrin R expression profile (Clarkson et al., 2014). However, the mechanism underlying regional differences in tonic firing is still unclear.

3.3.2 WT and KO animals undergo morphological changes associated with ageing

Although our findings illustrated that Purkinje cells from KO animals undergo morphological changes associated with age, the data also revealed a similar degeneration in WT mice. It was observed that the dendritic tree surface area is already decreased by 6 months of age in WT mice when compared to dendritic trees from 6 week old animals (Fig. 3.3a). These deterioration data were only significant in the posterior cerebellum indicating increased vulnerability in this area. An MRI study in SCA patients by Hulst et al. (2015) compared cerebellar degeneration in healthy ageing controls and patients suffering from cerebellar degeneration (a group consisting of patients with SCA6, SCA14, sporadic adult onset ataxia and autosomal dominant ataxia type III). The authors found that while degeneration in the anterior cerebellum is observed in both, healthy controls and patients, atrophy of the posterior cerebellar regions is only typical to patients with cerebellar degenerative diseases. The study, however, used patients with various degenerative cerebellar disorders, therefore data interpretation is more complicated.
Chapter 4

Characterisation and manipulation of primary cerebellar culture

4.1 Introduction

In the majority of neurodegenerative conditions, the underlying damage caused by the disease pre-dates the onset of symptoms (Przedborski et al., 2003). Furthermore, the symptoms usually manifest late in life (Przedborski et al., 2003). In spinocerebellar ataxia type 5 (SCA5), the patients are usually diagnosed around 30 years of age. To model such late-onset diseases and to speed up the drug discovery process not only animal models are used, but also cellular cultures (Schlachetzki et al., 2013). Primary rodent neuronal cultures, although limited in some aspects, are superior to cell lines and are expected to recapitulate the disease phenotype more accurately than tumor-derived cell lines, for instance (Datta, 2013).

Purkinje cells have one of the most elaborate dendritic networks of the entire nervous system. Since neuron shape and function are closely related (Darnell et al., 1999), any changes in Purkinje dendritic tree are expected to have functional outcomes. As Purkinje cell dendritic trees mostly develop postnatally (Armengol and Sotelo, 1991;
Chapter 4 Characterisation and manipulation of primary cerebellar culture

Tanaka, 2009, dissociated Purkinje cell cultures can be used to investigate the dendritic tree growth requirements (Kapfhammer, 2004).

However, it is important to note that the connection between the electrical activity of a cell and its dendritic development has been shown in multiple studies (Katz and Shatz, 1996; McAllister, 2000). For example, in the lateral geniculate nucleus, dendritic morphology is altered with monocular deprivation (Wiesel and Hubel, 1963). In the cerebellum, it has been demonstrated that blockade of N-methyl-D-aspartate receptor (NMDA) receptors reduces Purkinje cell dendritic trees (Vogel and Prittie, 1995). In Purkinje cell dissociated cultures Schilling et al. (1991) have also shown that cell activity block by tetrodotoxin (TTX) application impairs Purkinje cell dendritic tree development.

Calcium ions are universal signaling molecules in eukaryotic cells (Hudmon and Schulman, 2002) and their concentration is tightly regulated with a 20,000-fold difference across the membrane (Clapham, 2007). Calcium has been shown to regulate many aspects of cell’s life and death, due to its ability to alter function and localisation of its multiple protein binding partners (Clapham, 2007). The role of calcium in the dendritic formation has been examined in many studies usually by targeting one aspect of calcium signaling cascade (Finch and Augustine, 1998; Brozinick et al., 1999; Chen et al., 2009; Ashpole and Hudmon, 2011; Ashpole et al., 2012).

One of the major pathways through which calcium regulates dendritic tree development is by binding to the intracellular receptor calmodulin (Hudmon and Schulman, 2002). The most widely investigated binding partner is Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase II (CaMKII) (Schulman and Greengard, 1978) which upon calcium binding is relieved of autoinhibition and activated (Hudmon and Schulman, 2002; Clapham, 2007). \(\alpha\)CaMKII isoform is found to be widely expressed throughout the nervous system (Miller and Kennedy, 1985), while \(\beta\)CaMKII was found to be cerebellum-specific (Erondu and Kennedy, 1985). In the Purkinje cells of the posterior cerebellum, however, high levels of \(\alpha\)CaMKII isoform expression have been shown, demonstrating regional expression differences in the cerebellum (Wang et al., 2013).
Pharmacological manipulation of CaMKII activity has revealed some conflicting findings. CaMKII activation has been shown to be crucial for stabilizing dendritic tree and slowing down its growth in *Xenopus* retinotectal glutamatergic synapses (Wu et al., 1998). Similarly, constitutively active CaMKII was found to inhibit dendritic outgrowth in cortical neurons (Lohmann and Wong, 2005). However, in other types of cells, such as Neur2a cell line, overexpression of CaMKII was observed to promote dendritic growth (Goshima et al., 1993). In cerebellar granule cells, CAMKII block with KN-93 and PD098059 stimulated dendritic growth (Borodinsky et al., 2003). These conflicting findings make the interpretation of CaMKII results more complicated.

Purkinje cells exhibit calcium signaling in response to cellular inputs (Kitamura and Kano, 2013) and express multiple voltage-gated calcium channels (VGCCs) (Westenbroek et al., 1995). Furthermore, activity driven change in morphology has been shown in studies by Rakic and Sidman (1973) as well as Mason et al. (1997). Since T-type VGCCs get activated around resting membrane potential (Isope et al., 2012) and β-III spectrin knock-out (KO) mice have smaller Purkinje cells and thinner dendrites resulting in a higher resting membrane potential, it was hypothesized that T-type VGCCs would get activated earlier in these mice contributing to abnormal calcium signaling and subsequent changes in cell morphology.

In order to investigate the role of abnormal calcium signaling in dendritic tree morphology of Purkinje cells in β-III KO mouse, a dissociated Purkinje cell culture was established and evaluated for morphological and electrophysiological properties. Afterwards, the role of CaMKII in dendritic development was investigated by chronically blocking it in wild type (WT) and KO cerebellar cultures with KN62 (Tokumitsu et al., 1990). Finally, the effects of T-type calcium channel block were also evaluated by applying mibefradil to cerebellar cultures.
4.2 Results

4.2.1 Morphological and electrophysiological differences between WT and KO Purkinje cells in primary cerebellar culture

In order to accelerate drug discovery and investigate the role of calcium signaling in aberrant dendritic tree formation, it was crucial to establish a cell culture protocol, which would recapitulate previously observed differences between WT and KO Purkinje cells in SCA5 mouse model. It was observed that at 12 days in vitro (DIV) Purkinje cells from WT and KO cerebellar cultures had different morphological phenotypes (Fig. 4.1a). WT Purkinje cells displayed significantly larger cell surface in vitro (Fig. 4.1b). This is in line with previous findings that the KO Purkinje cell dendritic trees are smaller (Gao et al., 2011) and is shown in Fig. 3.2 and Fig. 3.3. Purkinje cells from KO cerebellar cultures, however, had significantly more dendritic branching points (Fig. 4.1c) and greater total dendritic length (Fig. 4.1d). Branch density, which was measured as the total amount of branching points per 100 µm of dendritic length, was also significantly increased in Purkinje cells from KO cerebellar cultures (Fig. 4.1e). These results indicate that at 12 DIV KO Purkinje cells have more disordered dendritic morphology with more branches and longer dendrites. However, they occupy a smaller surface area than WT Purkinje cells.

Purkinje cells in dissociated cerebellar cultures have been shown to be physiologically active with synaptic currents present (Schilling et al., 1991). To test whether the excitatory synaptic activity is present in our cultures, Purkinje cell activity was assessed by comparing firing frequency in the absence and presence of NBQX, an AMPA receptor blocker. Following drug application, tonically firing Purkinje cells were observed to undergo a significant decrease in firing frequency. This effect was observed in WT (Fig. 4.2a) and KO (Fig. 4.2b) cerebellar cultures and can be seen in sample traces in Fig. 4.3. These results indicate that excitatory input contributes to the firing profile of Purkinje cells in culture. Of note, firing frequency of WT Purkinje cells in the primary cerebellar culture was much smaller than in the cerebellar slices (6.0±0.9Hz
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and 29.4±1.8Hz, respectively).
Figure 4.1: Purkinje cells in KO primary cultures demonstrate abnormal cell morphology. a) Individual Purkinje cells from WT and β-III spectrin KO mice primary cerebellar cultures. Scale bar, 25 µm. WT Purkinje cell visualised by indirect fluorescence using rabbit anti-ITPR1 antibody, while KO Purkinje cell was visualised by direct fluorescence from intrinsically expressed GFP (**p=0.0053, Student’s t-test) b) WT Purkinje cells have significantly larger surface area c), d) Purkinje cells from KO cultures have more branching points (c, ****p< 0.0001, Student’s t-test) and greater total dendritic length (d, **p=0.0079, Student’s t-test) than WT cells e) KO Purkinje cells have more branching points per 100 µm of dendrites than WT Purkinje cells (****p< 0.0001, Student’s t-test, N=4-7, n=35-69).
When Purkinje cell electrophysiological recordings performed in the presence of NBQX were pooled for the cultures at 14 DIV, no difference was observed in spontaneous firing frequency between WT and KO Purkinje cells (Fig. 4.2c). Coefficients of variation (CV) for interspike interval (ISI) were also of similar values in WT and KO Purkinje cells (Fig. 4.2d). These findings suggest that the reduced spontaneous firing rate observed in KO mice acute slices (Perkins et al., 2010) is not recapitulated in dissociated cerebellar cultures.

In order to assess the effect of NBQX application on the cell firing mode, 1 or 10 µM NBQX was applied 100 seconds after breaking into the cell. It was observed that before NBQX application in both, WT and KO cerebellar cultures, the majority of cells were found to be either firing tonically or showed the presence of synaptic currents (Fig. 4.4d and e). After NBQX application approximately 36% and 53% of initially active Purkinje cells fell completely silent in WT (Fig. 4.4d) and KO (Fig. 4.4e) cultures, respectively. These results suggest that excitatory currents play important role in dissociated Purkinje cell firing profile.

Generally, it was observed that in cerebellar cultures, the proportion of spontaneously active Purkinje cells increased with culture age (data not shown), therefore the majority of spontaneous firing recordings were performed at 14 DIV or later. When comparing a few recordings from an earlier time point of culture development (7 DIV) it was also apparent that the firing frequency was higher at 14 DIV (Fig. 4.5a) similar to findings of other electrophysiologists working with cerebellar cultures (Schilling et al., 1991). However, no difference was identified in either cell excitability (Fig. 4.5c) or input resistance (Fig. 4.5d) in maturing cerebellar cultures.

Although no difference in spontaneous firing frequency was observed between WT and KO Purkinje cells in culture, tonically active KO Purkinje cells exhibited significantly higher cell excitability than active WT Purkinje cells, when passive Purkinje cell membrane properties were investigated as seen from sample traces in Fig. 4.6 and quantification in (Fig. 4.7a). However, there was no change in the cell input resistance (Fig. 4.7b). There was no difference in either cell output upon current injection.
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(Fig. 4.7c), or cell input resistance when silent Purkinje cells from WT and KO cerebellar cultures were analysed (Fig. 4.7d). Since the proportion of silent cells decreased with time in culture (Schilling et al., 1991), the silent state could potentially be an ‘immature’ cell state. These results would also suggest that even in dissociated cerebellar cultures KO Purkinje cells are more excitable than WT Purkinje cells, similar to previous findings from acute slices as seen in Chapter 3 (Perkins et al., 2010, 2016b).

Figure 4.2: NBQX application significantly decreases Purkinje cell firing rate in primary cerebellar cultures. a), b) Significant reduction is observed in firing frequency of Purkinje cells from WT (a, **p=0.0041, **p=0.0097, One-way ANOVA, N=2-5, n=6-11) and β-III spectrin KO (b, *p=0.0251, *p=0.0498, One-way ANOVA, N=2, n=7-10) cerebellar cultures c), d) No differences in either spontaneous firing frequency (c), or CV (d) is observed between Purkinje cells from WT and KO cerebellar cultures (N=4, n=16-21).
Figure 4.3: Sample traces of spontaneously firing Purkinje cells in 14 DIV dissociated cerebellar cultures of WT and KO mice before (top row) and after (bottom row) NBQX application.
Figure 4.4: a), b) and c) Sample traces of spontaneously tonically firing (a), bursting (b) and silent (c) Purkinje cell current clamp recordings, respectively, from 14 DIV dissociated cerebellar cultures after application of 10 µM NBQX d), e) Application of 10 µM of NBQX shifts firing mode of WT (d, **p=0.0035, Contingency table, N=8-10, n=25-53) and β-III spectrin KO (e, **p=0.0013, Contingency table, N=2-4, n=15-32) Purkinje cells by silencing them.

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**Figure 4.5:** Maturation of WT Purkinje cell culture. a) Spontaneous firing frequency increases with age in Purkinje cells from WT cultures in 10 µM NBQX (N=2-3, n=6-11) b) Current injection protocol used to produce input/output plots c), d) No differences in either cell excitability (c), or input resistance (d) is seen between tonically active WT Purkinje cells at 10 and 14 DIV in 10 µM NBQX (N=2-3, n=4-7).
Figure 4.6: Sample traces of Purkinje cells firing in WT and KO mice dissociated cerebellar cultures in response to injected depolarizing currents following the protocol shown in Fig. 4.5b and presented at 0, 150, 450 and 700 pA injection steps (top to bottom rows).
Figure 4.7: Spontaneously active Purkinje cells in KO cerebellar cultures show significantly elevated cell excitability. a) Spontaneously active tonically firing cells from KO cerebellar cultures fire at higher frequency than WT Purkinje cells following same current injection protocol (*p=0.0394, Two-way ANOVA, N=3-4, n=3-4) b) No differences in Purkinje cell input resistance observed c), d) Silent Purkinje cells show no differences in cell excitability (c) and input resistance (d) between Purkinje cells from WT and KO mice (N=4-8, n=12-22).
4.2.2 KO Purkinje cell morphology is determined autonomously

Given that Purkinje tree dendritic morphological abnormalities were recapitulated in dissociated cerebellar cultures, this *in vitro* system was used to decipher whether these defects were Purkinje cell autonomous. This was achieved by using different ratios of WT to KO cells in the same culture. The confocal image in Fig. 4.8a shows WT and KO Purkinje cells from a culture where the ratio of WT to KO cerebella was 1:7. From visual inspection, neither WT nor KO cell morphologies were observed to be influenced by each other. When the morphological properties of the cells were analysed using Neuronstudio, it was observed that the total number of branches (Fig. 4.8b) and the total dendritic length (Fig. 4.8c) was smaller in WT Purkinje cells when compared to KO Purkinje cells. Similarly, cell branch density of WT Purkinje cells was still significantly lower (Fig. 4.8d) despite the majority of culture contribution coming from KO cells. These results are identical to single genotype cultures in Fig. 4.1, suggesting that WT Purkinje cell morphology stays the same, regardless of presence and absence of KO Purkinje cells and majority of supporting cells being derived from the KO background. Likewise, the morphology of a KO Purkinje cell grown in culture with the majority of culture contribution coming from WT mice can be seen in Fig. 4.8e with no apparent changes in its dendritic tree when compared to a pure KO cerebellar culture (Fig. 4.1a). These results provide strong evidence that the abnormal Purkinje cell morphology in SCA5 is determined autonomously, advocating protein delivery as a viable approach to treating ataxia.
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Figure 4.8: Purkinje cell morphology is determined autonomously. a) Purkinje cells from WT (GFP, left column) and KO (anti-ITPR1 antibody, middle column) mice and their overlay (right column) co-cultured 1:7 WT to KO ratio. Scale bar, 25 µm. b), c) Total number of branches (b, ****p<0.0001, Student’s t-test) and total dendritic length (c, ***p=0.0005, Student’s t-test) are significantly smaller in WT Purkinje cells from co-cultures. d) Branch density is still significantly smaller in WT Purkinje cells regardless of majority culture contribution coming from KO animals (**p=0.0010, Student’s t-test, N=3, n=24-30). e) Individual KO Purkinje cell from a co-culture of WT and KO animals at 7:1 ratio. Scale bar, 25 µm.
4.2.3 KN62 application

As previously discussed, CaMKII plays a crucial role in calcium signaling and is an attractive pharmacological target. KN62, a CaMKII blocker, was applied at 10 µM concentration to WT cerebellar culture in a preliminary study, for either 5 or 12 days (Fig. 4.9a). Analysis of the dendritic tree revealed that a longer drug incubation had a greater effect on cell surface area (Fig. 4.9b), the total number of branches (Fig. 4.9c) and the total dendritic length (Fig. 4.9d). Very little effect was observed on branch density (Fig. 4.9e). Visual inspection of confocal images (Fig. 4.9a) reveals that the temporary application for 5 days at 7 DIV results in less complicated dendritic tree, however, the secondary and tertiary dendrites were still present. The chronic, 12-day incubation resulted in the formation of rudimentary dendritic tree, with mostly primary and secondary dendrites, and no thinner dendrites, suggesting severely impaired dendritic tree formation.

Similar results were observed following preliminary KN62 application (Fig. 4.10a) in β-III spectrin KO cultures. In this preliminary study WT and KO Purkinje cells showed similar surface area values in contrast to findings in Fig. 4.1a, which could potentially be explained by a small number of Purkinje cells analysed. Cell surface area (Fig. 4.10b) and total dendritic length (Fig. 4.10d) were inversely correlated with the length of KN62 incubation. The total dendritic length (Fig. 4.10d) and the number of branches (Fig. 4.10c) were reduced to similar levels observed in WT untreated cultures. Very little effect was observed on Purkinje cell branch density (Fig. 4.10e).

As previously noted, 10 µM concentration had quite a drastic effect on WT and KO Purkinje cells, and so the effects of the chronic application of lower KN62 concentrations were investigated on a WT Purkinje cell culture (Fig. 4.11). It was observed in a preliminary study that all applied concentrations had effects on cell surface (Fig. 4.12a), total number of Purkinje cell branches (Fig. 4.12b) and total dendritic length (Fig. 4.12c). None of the concentrations had any effect on the dendritic branch density (Fig. 4.12d). These results suggest the 2.5 µM KN62 concentration is still too high and lower concentrations need to be tested.
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Figure 4.9: Chronic 10 µM KN62 application has greater effects on WT Purkinje cell morphology than temporary application. a) Individual Purkinje cells from WT cerebellar culture at 12 DIV after short term (5d) and chronic (12d) KN62 application. Scale bar, 25 µm b), c) Gradual reduction in cell surface area (b) and total number of branches (c) is observed with prolonged periods of KN62 application d) Dendritic length is inversely correlated with the length of KN62 application e) KN62 had little effect on dendritic branch density (N=1, n=7-8).
Figure 4.10: Temporary KN62 application reduces branching in PC from KO cerebellar culture. a) Individual Purkinje cells from KO cerebellar culture after temporary (5d) and chronic (12d) application of 10 µM KN62. Scale bar, 25 µm b) KN62 application on KO cerebellar culture reduces cell surface area c) 5 days and 12 days of KN62 application reduces the total number of dendritic branches to the WT Purkinje cell level d) Chronic KN62 application causes greater reduction in total dendritic length e) No effect is observed on cell branch density (N=1, n=7-10).
Figure 4.11: Individual Purkinje cells after chronic application of KN62 at various concentrations. Scale bar, 25 µm.
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Figure 4.12: Chronic application of 2.5 μM KN62 has an effect on WT Purkinje cell morphology. a), b), c) Decrease in cell surface area (a), total number of dendritic branches (b) and dendritic length (c) is observed following KN62 application d) None of the tested KN62 concentrations had any effect on dendritic branch density (N=1-2, n=7-20).
4.2.4 Chronic mibefradil application improves KO Purkinje cell morphology

As deranged calcium signaling is expected to play a crucial role in abnormal dendritic tree morphology of β-III spectrin KO Purkinje cells, mibefradil, a T-type calcium channel blocker, was applied to WT and KO cerebellar cultures. As pharmacological compounds are likely to be delivered to the whole system rather than only the disease-affected cells, it was important to find the highest mibefradil concentration without any off-target effects i.e. without any consequences to the WT Purkinje cell morphology. Confocal images of individual Purkinje cells after chronic (12-day) incubation at various concentrations of mibefradil can be seen in Fig. 4.13. Analysis of WT Purkinje cell dendritic tree morphology revealed that 4 µM of mibefradil significantly decreased the cell surface area (Fig. 4.14a), the total number of dendritic cell branches (Fig. 4.14b) and total dendritic length (Fig. 4.14c). Contrarily, no effect was observed on dendritic branch density (Fig. 4.14d) after 4 µM mibefradil application. These results show that 0.5 µM and 2 µM mibefradil are potentially suitable concentrations for investigation of T-calcium channel block effect on Purkinje cells from KO cerebellar cultures.

Individual confocal images of KO Purkinje cells at 12 DIV following chronic application of mibefradil can be seen in Fig. 4.15a. It was discovered that the total number of dendritic branches (Fig. 4.15c) and the total dendritic length (Fig. 4.15d) were statistically indistinguishable from WT Purkinje cells, suggesting phenotypic improvement. In contrast, no effect on Purkinje cell surface area (Fig. 4.15b) was observed. Similarly, limited effect on branch density was detected (Fig. 4.15e). Nevertheless, these results suggest that chronic mibefradil application may have some effect on KO Purkinje cell dendritic tree morphology, suggesting that abnormal calcium signaling could be underlying some of the disease pathology.

In order to simulate late therapeutic intervention, mibefradil was applied at already established concentrations to WT cerebellar cultures at 7 DIV for 5 days only (Fig. 4.16a). This preliminary study showed no obvious changes in cell surface area (Fig. 4.16b), dendritic tree branching (Fig. 4.16c) or total dendritic length (Fig. 4.16d).
In contrast, late mibefradil application seemed to increase dendritic branch density (Fig. 4.16e), an effect which was not previously observed in chronic mibefradil application experiments.

When the same concentrations of mibefradil were applied to a KO cerebellar culture in a preliminary study (Fig. 4.17a), it was observed that Purkinje cell area was reduced when compared to KO and WT controls (Fig. 4.17b). Likewise, late mibefradil
application was found to decrease the total number of dendritic branches at both 0.5 µM and 2 µM (Fig. 4.17c). This reduction brought the KO Purkinje cells closer to the WT Purkinje cell values. In contrast, the dendritic length was not only reduced in the KO Purkinje cells (Fig. 4.17d), its observed values were indistinguishable from the WT controls. Finally, although some decrease was observed in dendritic branch density, these values were still larger than observed in the WT Purkinje cell controls (Fig. 4.17e).
Figure 4.15: Chronic mibefradil application improves KO Purkinje cell morphology.
(a) Individual Purkinje cells after chronic mibefradil application. Scale bar, 25 µm. 
b) No change in cell surface area is observed after chronic application of 0.5 µM and 2 
µM of mibefradil. c) After application of 0.5 µM and 2 µM of mibefradil, no significant 
difference is observed in total number of branches between KO Purkinje cells and 
WT control Purkinje cells (***p=0.0001, One-way ANOVA). d) Mibefradil application 
has no effect on total dendritic length (*p=0.0282, One-way ANOVA). e) Cell branch 
density is still significantly higher in KO Purkinje cells after 2 µM mibefradil application 
when compared to WT controls (****p<0.0001, *p=0.0335, One-way ANOVA, N=2-6, 
n=11-69).
Figure 4.16: Late mibebradil application has little effect on WT Purkinje cells. a) Individual Purkinje cells at 12 DIV from WT cerebellar culture after 5 day-long mibebradil application. Scale bar, 25 µm b) No effect on Purkinje cell surface area (b) or total number of branches (c) is observed after late mibebradil application at 7 DIV d) Late mibebradil application has no effect on total dendritic length of WT Purkinje cells in cerebellar culture e) Even late mibebradil application increases dendritic branch density at 0.5 µM and 2 µM (N=1).
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Figure 4.17: Late mibefradil application to KO cerebellar cultures alters Purkinje cell morphology. a) Individual Purkinje cells from KO cerebellar culture at 12 DIV after the last 5 days of mibefradil application. Scale bar, 25 µm b) Late mibefradil application reduces Purkinje cell surface area c) Total number of Purkinje cell dendritic branches is reduced after late mibefradil application d) Total dendritic length is decreased after mibefradil application e) Little effect is observed on dendritic branch density (N=1-6, n=5-69).
4.3 Discussion

4.3.1 Purkinje cells from β-III spectrin KO cerebellar cultures demonstrate higher cell excitability and an abnormal dendritic tree morphology

In summary, the results in this chapter demonstrated that β-III spectrin KO dissociated cerebellar cultures successfully recapitulate some of the Purkinje cell abnormalities seen in acute in vitro slices. This culture system therefore should be useful for the testing of potential therapeutic compounds. Here it was used to investigate the role of calcium signaling in Purkinje cell dendritic tree morphology.

The morphological analysis by 12 DIV revealed that dendritic trees of WT Purkinje cells had significantly bigger surface area than KO Purkinje cells, which is in line with findings in acute cerebellar slices (Gao et al., 2011; Perkins et al., 2016b). However, in cerebellar cultures, KO cells had significantly more branching points, as well as significantly increased dendritic length. In WT mice during the first postnatal weeks, Purkinje cells have been shown to send multiple primary dendrites, which are retracted during second postnatal week (Armengol and Sotelo, 1991). The same developmental process has been observed in vitro however multiple primary dendrites persist and are not retracted (Fujishima et al., 2012) suggesting differential pruning of Purkinje cell dendritic tree in vitro and in vivo.

As previously shown by Schilling et al. (1991), the majority of Purkinje cells from 14 DIV cerebellar cultures were either firing action potentials or showed the presence of excitatory synaptic currents. However, there was significant variation between cultures of the same genotype with some possessing 100% of Purkinje cells firing tonically, whereas in others only 50% and, in some instances, no Purkinje cells were found to be tonically active. This complicated any interpretation between WT and KO cultures and highlighted that multiple factors are likely to be critical for obtaining reliable electrophysiological data from dissociated cerebellar cultures, especially for KO Purkinje cells. Due to the timings the cultures were prepared from postnatal day 0 (P0) to P1
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pups, possibly affecting the culture viability. Also, the health of cultures was often not optimal with cultures only possessing a small number of Purkinje cells. Improving the quality of cerebellar cultures would be ideal in order to maintain the cells in culture for at least 21 days as the firing frequency of WT Purkinje cells should increase with culture maturation.

The electrophysiological analysis did not reveal any significant differences in firing rate frequency or CV between WT and KO Purkinje cells. However, tonically active Purkinje cells in β-III spectrin KO cultures were significantly more excitable when compared to WT Purkinje cells. These results are comparable to findings from acute in vitro slices that Purkinje cells from KO mice are more excitable (Perkins et al., 2010, 2016a). Input resistance, did not differ significantly between the genotypes, suggesting similar expression levels of leak channels and/or cell size.

4.3.2 WT and β-III spectrin KO Purkinje cell morphology is determined autonomously

Abnormalities in glial cells and not neurons have been suggested to lead to non cell-autonomous neurodegeneration (Custer et al., 2006; Lobsiger and Cleveland, 2007). For example, a study by Yazawa et al. (2005) demonstrated that α-synuclein expression in oligodendrocytes results in neuronal degeneration, while TAU pathology models in astrocytes causes degeneration of neurons (Forman, 2005). In the cerebellum, granule cells have been demonstrated to improve Purkinje cell survival and dendritic tree differentiation (Baptista et al., 1994) with neurotrophin signaling underlying the granule-Purkinje cell communication (Lindholm et al., 1993; Morrison and Mason, 1998). Bergmann glia, in turn, have been demonstrated to guide the Purkinje cell dendritic tree development towards the pial surface (Yamada et al., 2000) as well as granule cell migration (Rakic, 1971) and play a role in Purkinje cell morphogenesis (Tanaka et al., 2003). Non cell-autonomous Purkinje cell degeneration was also demonstrated in SCA7 (Custer et al., 2006).

The findings in this chapter reveal that even if the background is mainly from β-
III spectrin KO animals, Purkinje cells from WT animals maintain a WT morphology and *vice versa*. All of these findings suggest that abnormal Purkinje cell morphology observed in SCA5 is autonomously determined. Similar observations of cell autonomous degeneration have been made in Niemann-Pick disease type C (NPC) (Elrick et al., 2010) and SCA1 (Burright et al., 1995).

### 4.3.3 Chronic KN62 application has drastic effect on Purkinje cell morphology

Chronic (12 days) and late (5 days) application of KN62 had a similar effect on WT and β-III spectrin KO cerebellar cultures. It was observed that the longer KN62 was applied for, the less complicated were Purkinje cell dendritic trees. These results provide evidence that CaMKII plays a crucial role in dendritic tree development at all the developmental stages investigated.

Previous publications have demonstrated that prolonged CaMKII inhibition could be deleterious as CaMKII is also involved in neuronal survival (Ashpole et al., 2012). Indeed, βCaMKII KO mouse had pronounced ataxic phenotype (van Woerden et al., 2009). Global CaMKII KO has been shown to lead to greater cellular death (Waxham et al., 1996). In SCA5, however, cells from posterior cerebellum have been shown to be more vulnerable to cellular degeneration as seen in Chapter 3 (Perkins et al., 2016b). This vulnerability corresponds closely with the αCaMKII expression profile, with no observed expression in the anterior cerebellum and strong expression in the posterior cerebellar regions (Wang et al., 2013), further supporting deranged calcium signaling potentially underlying abnormal cell morphology. However, rather than targeting whole CaMKII signaling cascade, perhaps targeting alternative calcium signaling pathways like phospholipase C (PLC) would be beneficial in the future studies.
4.3.4 Chronic mibefradil application improves β-III spectrin Purkinje cell morphology

After identifying 0.5 µM and 2 µM mibefradil concentrations as not affecting normal WT Purkinje cell dendritic tree morphology, they were applied for 12 days on KO cerebellar cultures. It was observed that there was no effect on cell surface area. In contrast, the total number of dendritic branching and the total branch length were indistinguishable between WT and KO Purkinje cells. The branch density was also found to be slightly reduced. These results suggest that abnormal calcium signaling is implicated in SCA5 disease pathology and the manipulation of calcium levels in Purkinje may be able to improve cellular morphology.

The preliminary results from late mibefradil application revealed that 0.5 µM and 2 µM concentration had a significant effect on KO Purkinje cells. The total number of cell branches and the total dendritic length were both decreased reaching WT Purkinje cell levels. This suggests that later intervention may have a more beneficial effect on Purkinje cell dendritic tree. These preliminary results suggest that calcium signaling through T-type calcium channels could be important in the later phases of dendritic tree development rather than earlier in development.
Chapter 5

Carboxy-terminus of $\beta$-III spectrin elicits similar physiological effects to full length $\beta$-III spectrin

5.1 Introduction

Following the discovery in 1965 of adeno-associated virus (AAV) (Hoggan et al., 1966) and 25 years of basic AAV research, AAV viral therapy successfully entered pre-clinical studies in the early 90s (Hastie and Samulski, 2015). Early examples of its use include the expression of the cystic fibrosis transmembrane conductance regulator protein in the epithelium of rabbit lung tissue for 6 months following viral particle delivery (Flotte et al., 1993) and an improved behavioural outcome when human tyrosine hydroxylase was injected into denervated rat striatum (a mouse model for Parkinson’s disease) (Kaplitt et al., 1994).

AAV viruses have therefore shown therapeutic promise and are amongst the most widely used vectors in gene therapy (Daya and Berns, 2008) with 75% of current clinical trials utilising AAVs (Lentz et al., 2012; Kumar et al., 2016). The viral expression has
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been shown to be stable with some studies observing viral expression for years after delivery (Kaplitt et al., 1996; Niemeyer et al., 2009) and hosts generally show low immune reactions to the AAV (Zaiss et al., 2002; Bessis et al., 2004). Neutralising antibodies, however, have been found to decrease the viral product lifespan in liver and lungs (Daya and Berns, 2008). In contrast, in brain very little immune reaction is observed, probably due to the blood-brain barrier (Lo et al., 1999; Daya and Berns, 2008). Furthermore, immune reaction also seems to depend on the serotype of AAV used (Daya and Berns, 2008) and the mechanism of the AAV delivery into the organism (Brockstedt et al., 1999).

The expression profile and the type of cells transduced also depend on the AAV serotype (McCown et al., 1996; Daya and Berns, 2008; Zincarelli et al., 2008). AAV2 was first isolated from adenovirus preparations and has become the most widely used AAV serotype (Wu et al., 2006; Buning et al., 2008). AAV2 infects cells after binding heparan sulfate proteoglycan on the cell surface (Summerford and Samulski, 1998; Van Vliet et al., 2008) and it has been shown to have high efficiency of viral transduction in the central nervous system (Bartlett et al., 1998; Davidson et al., 2000; Xu et al., 2001). Other AAV serotypes, of which there have been around one hundred discovered to date (Wu et al., 2006; Kwon and Schaffer, 2008), express different viral capsid proteins resulting in infection of various cell types and tissue tropism (Lentz et al., 2012). Pseudotype AAVs are also being produced by expressing viral genome and protein capsids from different AAVs, resulting in the ability to target specific cells (Rabinowitz et al., 2002; Burger et al., 2004; Zincarelli et al., 2008) and avoiding AAV detection by neutralising antibodies (Hauck and Xiao, 2003). Incorporating cell specific promoters achieves further cell specificity in recombinant AAVs (rAAVs) (Kim et al., 2015). In the cerebellum, AAV1 (Xia et al., 2004; Kim et al., 2015), AAV2 (Kaesmacher et al., 2000) and AAV5 (Alisky et al., 2000) have all shown efficient Purkinje cell transduction. In this study the chimeric AAV1/2 viral particles have been used with commonly used AAV2 packaging signals (inverted terminal repeats, ITR), AAV1/2 protein capsids and the Purkinje cell specific enhanced synapsin promoter.
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The major limiting factor in the usage of AAV vectors is their low packaging capacity (up to 4.7 kb), with bigger inserts resulting in a decreased packaging efficiency (Dong et al., 1996). When AAV insert exceeds 5 kb, truncated insert versions of various lengths were shown to be packaged and to recombine into full length proteins in a destination cell, however this occurs with a low efficiency (Wu et al., 2010; Lentz et al., 2012). An alternative approach to overcome the size limitation is using transsplicing AAVs, where the gene of interest is split over two vectors (Yan et al., 2000). In this study a novel method of delivery a part of the protein was used.

rAAV production in the lab generally relies on the transient transfection of Human Embryonic Kidney (HEK293FT) cells with multiple plasmids (Blouin et al., 2004). The AAV viral production protocol followed in this thesis requires transfection of HEK293FT cells with 4 plasmids simultaneously: AAV plasmid containing the cDNA of protein of interest and AAV2 packaging signals, pFΔ6 (adenovirus-helper plasmid), pRV1 (AAV2 rep and cap sequences) and pH21 (AAV1 rep and cap sequences) (McClure et al., 2011). Although calcium phosphate transfection technique is considered to be cost-effective (Kim and Eberwine, 2010), it was also investigated which transfection method maybe be the most appropriate for viral production.
5.2 Results

5.2.1 C-trm β-III has a stable expression in HEK293FT cells and localises to the plasma membrane

cDNA of the full length (FL) β-III spectrin is 7.2 kb, encompassing actin binding domain in the N-terminus, 17 spectrin repeats, as well as ankyrin-binding and pleckstrin homology (PH) domains in the C-terminus (Fig. 5.1, top row). As the AAV vector system is only able to accommodate inserts up to 4.7 kb in length, an alternative cloning approach was needed. N-terminus (N-trm, Fig. 5.1, second row), middle (Fig. 5.1, third row) and C-terminus (C-trm, Fig. 5.1, bottom row) of β-III spectrin were cloned into the myc-tagged pRK5 vector.

Western blot analysis showed that the strongest bands had the expected molecular weights (Fig. 5.2), which were 86.2 kDa for N-trm, 175.2 kDa for middle β-III spectrin fragment, 95.2 kDa for C-trm and 272.2 kDa for FL β-III, accounting for 1.2 kDa myc tag. However, N-trm, middle and FL β-III spectrin had other fainter bands.

The localisation of β-III spectrin fragments expressed from the myc-tagged pRK5 vector was evaluated by taking confocal images of transfected HEK293FT cells (Fig. 5.3). It was observed that only FL and C-trm β-III spectrin fragments were expressed at the

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**Figure 5.1:** Schematic of FL β-III spectrin and fragments cloned into pRK5 and pL7-mCherry expression vectors. FL β-III spectrin has the actin binding domain, ankyrin binding domain (spectrin repeat 15), PH domain and 17 spectrin repeats. N-terminus β-III spectrin (N-trm) contains actin binding domain and the first four spectrin repeats. Middle β-III spectrin fragment carries spectrin repeats 2 through to 15 with ankyrin binding domain. C-terminus β-III spectrin (C-trm) contains spectrin repeats 13 to 17, ankyrin binding and PH domain.
Carboxy-terminus of β-III spectrin elicits similar physiological effects to full length β-III spectrin

**Figure 5.2:** Expression of myc-tagged proteins. Western blot analysis of cell homogenates from HEK293FT cells transfected with myc-tagged N-terminus (N-trm), middle, C-terminus (C-trm) and full length (FL) β-III spectrin fragments. Homogenate from untransfected (UT) cells is included as a negative control. Nitrocellulose membrane was probed with mouse antibody raised against myc tag (N=2).

cell membrane. In contrast, N-trm was actin-associated and middle fragment appeared to be located in the cytoplasm.

However, it was also important to address whether the fragments of β-III spectrin would exert any dominant negative effect on FL β-III spectrin as this would prevent any therapeutic benefit. To achieve this the same fragments were cloned into a different vector system (pL7-mCherry) and co-expressed with myc-tagged FL β-III spectrin.

Western blot analysis of mCherry-tagged fragments showed similar results (Fig. 5.4). As mCherry weight is 30 kDa, the expected molecular weights for the fragments were 115 kDa for N-trm, 204 kDa for middle fragment, 124 kDa for C-trm and 301 kDa for FL β-III spectrin. As with myc-tagged proteins, all fragments had some fainter lower molecular weight bands, potentially showing protein degradation products.

Confocal image analysis revealed that, similar to myc-tagged β-III spectrin proteins, mCherry-tagged FL β-III spectrin was expressed at the plasma membrane (Fig. 5.5). mCherry-tagged C-trm β-III also had higher expression regions at the membrane, however some expression was seen in the cytoplasm. Of note, expression of C-trm in both vector systems was seen to induce cellular filopodia and protrusion outgrowths, which were not seen in FL β-III spectrin transfected cells. mCherry-tagged N-trm was again found to exhibit an actin cytoskeletal association and the middle fragment was located within the cytoplasm.
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**Figure 5.3:** Cellular distribution of myc-tagged full length (FL), N-terminus (N-trm), middle and C-terminus (C-trm) of β-III spectrin fragments expressed in HEK293FT cells. Expression profile was visualised by the indirect fluorescence using rabbit anti-C-trm (FL and C-trm) and mouse anti-myc (N-trm and middle) antibodies (N=2). Scale bar, 15 µm.
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Figure 5.4: Expression of mCherry-tagged proteins. Western blot analysis of cell homogenates from HEK293FT cells transfected with mCherry-tagged N-terminus (N-trm), middle and C-terminus (C-trm) of β-III spectrin fragments. HEK293FT cells transfected with full length cDNA (FL), full length cDNA in wrong orientation (FL WO) and untransfected (UT). Homogenate from untransfected (UT) cells included as a negative control. Nitrocellulose membrane probed with the antibody raised against DsRed (N=2).
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**Figure 5.5:** Cellular distribution of full length (FL), N-terminus (N-trm), middle or C-terminus (C-trm) of mCherry-tagged β-III spectrin fragments expressed in HEK293FT cells. Expression profile visualised by either direct fluorescence from mCherry tag (FL and N-trm) or indirect fluorescence using rabbit anti-DsRed antibody (middle and C-trm) (N=2). Scale bar, 15 µm.
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5.2.2 C-trm does not have a dominant-negative effect on FL β-III spectrin

After successfully expressing FL β-III in myc-tagged and mCherry-tagged vectors, their localisation was evaluated following co-transfection. Confocal microscopy analysis (Fig. 5.6a) revealed that both β-III spectrin fragments were expressed at the cell membrane. Furthermore, both proteins co-localised (Fig. 5.6b) with Pearson’s coefficient reaching 0.98 and Costes P-value of 1.00 indicating significant co-localisation (Fig. 5.6c).

To investigate whether myc-tagged and mCherry-tagged β-III spectrin fragments

![Figure 5.6: Myc-tagged and mCherry-tagged full length β-III spectrin fragments co-localise at the cell membrane. a) HEK293FT cells co-transfected with myc-tagged full length (green, left) and mCherry-tagged full length β-III spectrin (red, middle) and their overlay (right). Mouse anti-myc and rabbit anti-DsRed antibodies used for fragment detection. Scale bar, 15 µm b) 2D intensity histogram of green and red pixels c) Co-localisation values indicate significant co-localisation of myc-tagged and mCherry-tagged FL β-III spectrin (N=2).](image-url)
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have any dominant negative effect on WT FL β-III spectrin, co-transfections were performed. First, m-Cherry tagged β-III spectrin fragments were co-transfected with myc-tagged FL β-III spectrin (Fig. 5.7), while myc-tagged β-III spectrin fragments were co-transfected with mCherry-tagged FL β-III spectrin (Fig. 5.8).

Following co-transfection with N-terminus, (Fig. 5.7, top row and Fig. 5.8, top row) FL myc-tagged or mCherry-tagged β-III spectrin was found to localise mostly at the cell membrane (Fig. 5.7, top row, middle and Fig. 5.8, top row, middle). N-terminus, however, was seen mostly in the cytoplasm (Fig. 5.7, top row, left and Fig. 5.8, top row, left), consistent with previous single transfections (Fig. 5.3 and Fig. 5.5).

Similar to single transfection experiments (Fig. 5.3 and Fig. 5.5), myc-tagged (Fig. 5.7, middle row, left) and mCherry-tagged (Fig. 5.8, middle row, left) middle β-III spectrin fragments had classic cytoplasmic localisation, when coexpressed with FL protein. In both experiments, FL β-III spectrin was localising at the membrane (Fig. 5.7, middle row, middle and Fig. 5.8, middle row, middle) indicating no dominant negative effect.

As seen previously (Fig. 5.3 and Fig. 5.5), C-trm β-III spectrin localised mainly in the membrane (Fig. 5.7, bottom row, left and Fig. 5.8, bottom row, left) when co-expressed with FL β-III spectrin. FL β-III spectrin was found mostly at the membrane (Fig. 5.7, bottom row, middle and Fig. 5.8, bottom row, middle) indicating no dominant negative effect. Of note, some cytoplasmic staining (Fig. 5.8, bottom row, middle) was observed. However, expression of C-trm alone (Fig. 5.3 and Fig. 5.5) or together with FL protein (Fig. 5.8) results in altered cell morphology with numerous protrusions.
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Figure 5.7: Myc-tagged full length $\beta$-III spectrin (green, middle column) co-transfected with mCherry-tagged N-terminus (N-trm, top row), middle (middle row) and C-terminus (C-trm, bottom row) $\beta$-III spectrin fragments and expressed in HEK293FT cells. Mouse anti-myc and rabbit anti-DsRed antibodies used for fragment detection (N=3). Scale bar, 20 µm.
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**Figure 5.8:** mCherry-tagged full length β-III spectrin (red, middle column) co-transfected with myc-tagged N-terminus (N-trm, top row), middle (middle row) and C-terminus (C-trm, bottom row) β-III spectrin fragments and expressed in HEK293FT cells. Mouse anti-myc and rabbit anti-DsRed antibodies used for fragment detection (N=3). Scale bar, 20 µm.
5.2.3 C-trm β-III spectrin increases Na\textsubscript{v} currents in hippocampal pyramidal neurons

As C-trm β-III spectrin was found to localise at the cell membrane, where functional FL β-III spectrin is also found, electrophysiological recordings were performed to investigate its functional effects. Full length β-III spectrin has been shown to increase sodium currents in dissociated hippocampal cultures (Clarkson et al., 2014), therefore to be of a therapeutic use, C-trm would be required to elicit a similar effect. Representative sodium current traces can be seen in Fig. 5.9, evoked with a series of 50 ms depolarization steps from a holding potential of -90 mV to potentials ranging from -80 to +20 mV in 10 mV increments (Fig. 5.9, bottom right). C-trm β-III (Fig. 5.9, bottom left) can be seen to increase sodium currents when compared with untransfected (UT) neurons (Fig. 5.9, top left) to similar levels of full length β-III spectrin (Fig. 5.9, top right).

**Figure 5.9:** Whole-cell sodium current recordings from pyramidal neurons at 7 days *in vitro* (DIV). Representative traces from untransfected (UT) or neurons transfected with either full length (FL) or C-terminus (C-trm) β-III spectrin fragments. Bottom right, voltage step protocol used in obtaining whole-cell current recordings.
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Quantification of Na\textsubscript{v} current analysis revealed FL and C-trm β-III have identical current-voltage relationships (Fig. 5.10a), suggesting that introduction of part of the protein would be enough to increase sodium currents and elicit a desired functional effect. Both, FL and C-trm had significantly higher sodium currents when compared to untransfected neurons from -30 mV to +20 mV. When normalized peak currents were compared between different fragments (Fig. 5.10b) no significant difference was observed between FL and C-trm β-III spectrin confirming similar physiological effects.

**Figure 5.10:** C-terminus (C-trm) β-III spectrin increases pyramidal neuron sodium currents to full length (FL) β-III spectrin levels. a) Voltage-current relationship reveals no difference in channel kinetics observed between FL and C-trm β-III spectrin nucleofected neurons. FL and C-trm significantly increased sodium currents from -30 mV to 10 mV when compared to untransfected (UT) neurons (Two-way ANOVA) b) The peak amplitude of Na\textsubscript{v} currents from FL and C-trm β-III spectrin nucleofected neurons at -10 mV step normalized to UT cell currents. C-trm significantly enhances sodium current in pyramidal neurons to levels similar to WT β-III spectrin (N=6, n=20).


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5.2.4 Nucleofection is the most efficient transfection method for single plasmid introduction

Although calcium phosphate is a widely used and cost-effective transfection method in the production of AAV particles, experiments were carried out to find out whether it is optimal for obtaining a high viral titre. In order to investigate the efficiency of calcium phosphate transfection method and compare it with other widely used transfection methods the co-transfection efficiency of multiple vectors was quantified.

In the first experiment, HEK293FT cells were transfected with a single plasmid expressing nuclear yellow fluorescent protein (YFP) using calcium phosphate, Lipofectamine, X-tremeGENE and nucleofection (Fig. 5.11). Transfection efficiency was then analyzed by counting the percentage of cells expressing the reporter protein after staining all nuclei with DAPI. Preliminary results showed that nucleofection was more efficient at HEK293FT cell transfection when compared with other methods, with calcium phosphate being the least efficient (Fig. 5.12). Lipofectamine and X-tremeGENE had similar transfection efficiency in HEK293FT cells which were slightly higher when compared to calcium phosphate. This experiment was only performed once to check which transfection method is superior and whether it would be worth improving viral production protocol. As calcium phosphate yielded adequate transfection efficiency at much lower costs, experiment was not repeated.
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Figure 5.11: Representative confocal images of nuclear-YFP transfection (YFP), with all HEK293FT nuclei visualised (DAPI) and their overlay following calcium phosphate, Lipofectamine, X-tremeGENE and nucleofection transfection methods. Scale bar, 50 μm.
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Figure 5.12: Percentage of HEK293FT cells transfected with nuclear YFP fluorophore using different transfection methods reveals that nucleofection transfects more cells when compared to calcium phosphate, Lipofectamine and X-tremeGENE. Calcium phosphate transfected the smallest proportion of cells (N=1).
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5.2.5 Nucleofection is the least suitable transfection method for quadruple plasmid delivery

As viral production relies on cells transfected with 4 plasmids simultaneously, it was investigated which transfection method is the most reliable when multiple plasmids are required. A confocal image of a HEK293FT cell, expressing 4 fragments simultaneously can be observed in Fig. 5.13 following calcium phosphate transfection method. When all the cells transfected were counted in the preliminary study, it was observed that calcium phosphate, lipofectamine and X-tremeGENE have very similar transfection profile: roughly a quarter of transfected HEK293FT cells expressed 1, 2, 3 and 4 plasmids (Fig. 5.14a). However, following nucleofection, hardly any HEK293FT cells expressed 4 plasmids (Fig. 5.14a and Fig. 5.14e). Further data analysis revealed, that the biggest proportion of calcium phosphate transfected cells only expressed 1 plasmid (Fig. 5.14b), which was more when compared to other transfection methods. No difference was observed when the proportion of cells expressing 2 plasmids was compared (Fig. 5.14c). Furthermore, nucleofection transfected a bigger proportion of cells with 3 plasmids (Fig. 5.14c), however, hardly any cells expressed 4 plasmids after quadruple transfection (Fig. 5.14e). These results appear to indicate that nucleofection may be the least suitable method for a 4 plasmid transfection. This experiment was only performed once to identify whether calcium phosphate performed to a similar standard compared to the other transfection methods in multiple plasmid transfection protocol and was not repeated.
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**Figure 5.13:** HEK293FT cell expressing nuclear YFP, extra-nuclear green fluorescent protein (GFP), ubiquitous DsRed and myc-tagged C-trm β-III spectrin plasmids using calcium phosphate (N=2). Scale bar, 20 µm.
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Figure 5.14: Quantification of plasmid expression after transfecting HEK239FT cells with 4 plasmids. a) 4 plasmid transfection efficiency comparison using different nucleofection methods reveals that only a small proportion of nucleofected cells were expressing all 4 plasmids b) After 4 plasmid transfection, significantly bigger proportion of calcium phosphate transfected cells was expressing just one plasmid when compared to other transfection methods c) Similar proportion of transfected cells were expressing 2 plasmids across different transfection methods d) A bigger proportion of nucleofected cells were expressing 3 plasmids when compared with other transfection methods e) The smallest proportion of transfected cells expressing 4 plasmids was following nucleofection (N=1).

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5.3 Discussion

5.3.1 C-trm stable expression in HEK293FT cells

Due to the AAV system’s packaging limitations (4.7 kb) it was not feasible to clone the FL \( \beta \)-III spectrin (7.3 kb) directly. As an alternative approach, experiments in this chapter showed that only a part of \( \beta \)-III spectrin could potentially be used for gene therapy. Out of all fragments cloned, only C-trm \( \beta \)-III spectrin was found to localise at the membrane just like FL \( \beta \)-III spectrin. However, the expression profile seemed to differ slightly depending on the destination vector - myc-tagged C-trm was found to localise cleanly at the membrane (Fig. 5.3), while mCherry-tagged C-trm \( \beta \)-III spectrin had also some cytoplasmic presence (Fig. 5.5). That could possibly be due to the much bigger protein tag coming from pL7-mCherry vector. Myc tag localises at the N-terminus, while mCherry tag is at the C-terminus, which might also alter the cellular localisation by preventing the cloned fragments from interacting with their regular binding partners. Additionally, C-trm \( \beta \)-III spectrin expression initiated formation of additional cellular protrusions.

5.3.2 C-trm \( \beta \)-III spectrin fragment does not have a dominant negative effect on the FL \( \beta \)-III spectrin

Spinocerebellar ataxia type 5 (SCA5) is thought to be brought about by a dominant negative effect, therefore it was crucial to find a fragment of \( \beta \)-III spectrin which was unlikely to elicit a dominant negative effect on WT \( \beta \)-III spectrin. Two different vector systems were used: myc-tagged pRk5 and pL7-mCherry. When each fragment (N-trm, middle and C-trm) was co-transfected with FL \( \beta \)-III spectrin (Fig. 5.7 and 5.8), it was clear that the localisation of the FL \( \beta \)-III spectrin had strong staining at the plasma membrane suggesting no dominant negative effect.
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5.3.3 C-trm β-III spectrin has similar physiological effect as FL β-III spectrin

Our lab had previously shown that FL β-III spectrin significantly increases Na\textsubscript{v} current in dissociated hippocampal cultures (Clarkson et al., 2014). This established system, therefore, was used to examine potential physiological effects of C-trm β-III spectrin. It was observed that primary rat hippocampal neurons nucleofected with C-trm β-III spectrin had increased sodium currents (Fig. 5.9). Furthermore, the quantification revealed that peak currents of FL and C-trm β-III spectrin were indistinguishable (Fig. 5.10). C-trm β-III encompasses ankyrin binding domain and β-III spectrin has been demonstrated to be crucial for normal ankyrin R cellular distribution (Clarkson et al., 2014). As spectrin/ankyrin R complex is hypothesised to be important for Na\textsubscript{v}1.1 and Na\textsubscript{v}1.6 stabilisation (Perkins et al., 2016b), the finding that C-trm β-III spectrin increases sodium currents suggests functionality of the cloned fragment.

5.3.4 Nucleofection is the most efficient transfection method for single, but possibly not quadruple transfection

Viral production relies on transient HEK293FT cell transfection with 4 different fragments, therefore it was important to investigate which method of DNA transfection would optimise viral titre. Initial experiments revealed that transfection with single plasmid was the most efficient with nucleofection method as nearly 70% of cells expressed the transfected protein (Fig. 5.11 and 5.12), a much larger proportion when compared to calcium phosphate, Lipofectamine and X-tremeGENE. This transfection mechanism is proposed to be more efficient due to direct DNA delivery into the nucleus rather than cytoplasm by introducing an electric shock to the cell (Distler et al., 2005). However, nucleofection is much more costly when compared to other transfection methods, as well as additional equipment is needed such Nucleofector apparatus, which makes its use quite complicated on a regular and large scale basis. It was also observed that calcium phosphate transfected the smallest proportion of cells (Fig. 5.12). Calcium phosphate, which was first developed by Graham and van der Eb (1973), relies on
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the precipitate formation between DNA and calcium phosphate (Chen and Okayama, 1987). Calcium phosphate then facilitates the binding of the complex to the cell membrane and then the DNA is internalised by endocytosis (Chen and Okayama, 1987). Calcium phosphate in our lab has been observed to deliver variable results depending on temperature, pH of solutions and health of cells.

When quadruple transfection experiments were performed, just a quarter of the cells had all 4 transfected plasmids following nucleofection. In general it was observed that roughly a quarter of cells were expressing 1, 2, 3 or 4 plasmids after calcium phosphate, Lipofectamine and X-tremeGENE. Lipofectamine and X-tremeGENE methods rely on positively charged lipids to bind DNA and negatively charged cell membrane with neutral lipids assisting the fusion with cell membrane (Dalby et al., 2004). Results in this chapter indicate that only a quarter of such lipid-DNA complexes contained all 4 plasmids.

As calcium phosphate transfection resulted in a similar proportion of 4-plasmids expressing cells, this transfection method was adopted for viral production. However, as it will be reported in Chapter 6, the first batch of virus harvested was found to have a very low titre. To improve the protocol subsequent batches were harvested from a greater number of HEK293FT cell plates. In the future it may be worthwhile to re-visit whether there is a better method for multiple DNA transfections as, unfortunately, the YFP and GFP fluorophores used had quite a strong bleed-through, resulting in some difficulty to accurately quantify co-expression. Furthermore, transfection methods utilising cationic liposomes (Lipofectamine and X-tremeGENE) have also been demonstrated to rely heavily on cells undergoing mitosis (Tseng et al., 1999), therefore lengthening the time window between transfection and cell fixation may be beneficial.

Although, nucleofection has been suggested to be a quite gentle transfection method (Distler et al., 2005), the transfection protocol required at least 1×10⁶ cells to be transfected in one go. To achieve similar cell density when comparing with other transfection methods, seeding twice as many cells in 12-well tissue plate rather than 6-
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well was required. Analysis of the results is therefore further complicated, since all cells were fixed 24 hours after transfection. That meant 48 hours after plating for calcium phosphate, Lipofectamine and X-tremeGENE and only 24 hours for nucleofection, as nucleofection is performed at the same time as cell plating. To achieve more definitive results experimental design should be improved in the future.
Chapter 6

Introduction of C-terminus β-III spectrin via stereotaxic surgery

6.1 Introduction

Despite massive advances in medicine and research over the last decades, diseases of the nervous system are particularly challenging to treat to this day. These disorders are usually chronic and extremely complex, hindering the drug discovery process (Varma et al., 2008; Marques et al., 2011; Stanzione and Tropepi, 2011). Early diagnosis of neurodegenerative conditions is also difficult due to cellular, molecular and anatomical abnormalities occurring much earlier than the onset of first symptoms (Bateman et al., 2012; Aylward et al., 2013).

Even after discovery of therapeutically promising active substances, patients can only benefit from them if the drug is able to cross the blood-brain barrier and reach its target (Carvey et al., 2009; Palmer, 2011; Saraiva et al., 2016). This often limits the systemic delivery of potentially neuroprotective substances to the brain. Furthermore, systemic drug delivery to the brain can also result in unwanted side effects (Nutt and Wooten, 2005). As different neurodegenerative conditions affect different brain regions, localised active substance delivery is therefore preferable (Obeso et al., 2000; Wichmann and DeLong, 2003).
Since the first successful gene therapy experiment in which growth hormone was returned to growth-hormone deficient rats (Hammer et al., 1984), gene therapy has been explored as a potential treatment in nervous system disorders. In the last few decades, various types of viruses have been utilised to deliver genes in order to modulate protein expression profile in various cell types. In recent years adeno-associated viruses (AAVs) have been the workhorse for gene delivery (Daya and Berns, 2008). Stable gene product expression (Kaplitt et al., 1994), limited immune reaction (Zaiss et al., 2002) and targeted viral delivery make AAVs attractive candidates for the gene therapy.

AAV gene therapy has been widely used in mice models, primates (Hadačzek et al., 2010) and in some human clinical trials (Varenika et al., 2009; Mingożzi and High, 2011). Some modest successes have been achieved in neurodegenerative conditions. In one Parkinson’s disease clinical trial the delivery of glutamic acid decarboxylase into subthalamic nucleus led to improved motor ratings in patients (Kaplitt et al., 2007). In mice, delivery of glial cell line derived neurotrophic factor (GDNF) 4 weeks after intrastriatal lesion modeling Parkinson’s disease, showed motor improvement and promoted dopaminergic neuron survival (Wang et al., 2002).

In the cerebellum, AAV gene therapy has been tested in various neurodegenerative cerebellar disorders. In globoid cell leukodystrophy, which is caused by the deficiency of galactocerebrosidase enzyme (GALC), delivery of AAV2/5-GALC construct under control of CMV early enhancer/chicken β actin gene (CβAG) promoter at postnatal day 3 (P3), resulted in increased Purkinje cell density and prevented Purkinje cell dendritic tree degeneration (Lin et al., 2015).

In the ataxia field, most mice models which adopted the AAV gene therapy approach were models of spinocerebellar ataxia 1 (SCA1). SCA1 is a dominant, polyglutamine expansion disorder in which aggregation of mutant protein ataxin-1 are formed and Purkinje cell loss is observed (Skinner et al., 1997) (also, Section 1.3.1) and a mouse model of SCA1 was published by Burright et al. (1995). A publication by Rodriguez-Lebron et al. (2013) showed that delivery of AAV-micro RNA (miRNA) construct into these SCA1 mice successfully targeted mutant ataxin-1, reducing its aggregation.
Another study showed that injection of silencing RNA into the same SCA1 mouse model at 7 weeks of age, targeting ataxin-1, improved their motor performance and increased thickness of the molecular layer, suggesting reversed neurodegeneration (Xia et al., 2004). AAV2 Voltage- and Ca\(^{2+}\)-activated K\(^+\) (BK) channel construct delivery into cerebellum at 4 weeks of age of SCA1 mutant animals shifted Purkinje cell firing mode to more cells firing tonically, increased Purkinje cell firing frequency towards wild type (WT) animals and resulted in a modest increase in Purkinje cell density (Dell’Orco et al., 2015).

SCA2, the second most common SCA (Schöls et al., 2004) is also caused by polyglutamine tract (polyQ) expansion (Imbert et al., 1996) (also, Section 1.3.1). Deranged calcium signaling is implicated in SCA2 pathology (Kasumu and Bezprozvanny, 2012a) and in SCA2 mouse model by Huynh et al. (2000) due to sensitization of inositol 1,4,5-trisphosphate receptor (ITPR). Delivery of mouse inositol 1,4,5-phosphatase (Inpp5a) enzyme (5PP) was found to suppress ITPR mediated Ca\(^{2+}\) release by reducing its response to stimuli (Kasumu et al., 2012b). This approach restored Purkinje cell firing rate and decreased coefficient of variation (CV) of interspike interval (ISI) towards WT Purkinje cell levels after injection at 7 weeks of age (Kasumu et al., 2012b).

To evaluate what effect expressing the C-terminus of β-III spectrin had on Purkinje cell morphology, survival and electrophysiological properties, as well as motor performance of β-III spectrin knock-out (KO) mice, viral particles were stereotaxically injected at different ages.
6.2 Results

6.2.1 Stereotaxic surgery per se has no effect on mice motor performance

Before carrying out viral delivery of β-III spectrin, control surgeries were performed by injecting pre-existing CβAG-green fluorescent protein (GFP) AAV 1/2 viral particles into WT animals. This was carried out to reveal whether the procedure itself had any effect on mice motor performance. The mice were tested 1 month before (at 3 months of age) and one month after the surgery (5 months of age). The coordinates for cerebellar stereotaxic surgery were based on previous publication by White et al. (2011) with the injection targeting lobules III and IV in the anterior cerebellum and lobule X in the posterior cerebellum. Resulting AAV expression can be seen in the confocal image in Fig. 6.1a.

The evaluation of WT mice performance on the rotarod before and after control surgery (Fig. 6.1b) revealed no effect on their motor performance at 3 and 5 rpm speeds, however there was a small but significant decrease in latency to fall off at 10 rpm. Analysis of hind-paw slips made during an elevated beam test showed no consequence of injection on motor coordination (Fig. 6.1c).

Gait analysis similarly showed no differences in either stride length (Fig. 6.1d) or width (Fig. 6.1e) before and after the surgery. As a routine well-being check mice weight was also monitored (Fig. 6.1f) and no differences were found following the CβAG-GFP surgery.
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**Figure 6.1:** Control stereotaxic surgery has no effect on WT mice motor performance.  
a) Confocal image of sagittal cerebellar slice from WT mouse 1 month after C$\beta$AG-GFP viral construct injection at 3 months of age. Scale bar, 500 µm (*p=0.0475, Two-way ANOVA)  
b) Rotarod performance of WT mice before and after the surgery showed no significant effect of the stereotaxic surgery at 3 and 5 rpm, with slight motor deterioration at 10 rpm  
c) Elevated beam test before and after surgery showed no significant changes  
d) Mice gait analysis showed no changes in stride length or width following the control injection  
e) Body weight analysis revealed no change after the surgery (N=4).
6.2.2 Low-titre AAV1/2-eSyn-C-trm-IRES-eGFP localises in posterior cerebellum of β-III spectrin KO mice

After production of the first viral batch, AAV1/2-eSyn-C-trm-IRES-eGFP viral particles were delivered at 3 months of age into WT and β-III spectrin KO mice and the animals were observed for 5 months. At 8 months of age the mice were sacrificed and the viral expression profile was evaluated by analysing sagittal cerebellar slices. Although the viral expression was found to be quite low by confocal microscopy (Fig. 6.2), analysis revealed that more Purkinje cells from WT mice expressed AAV1/2-eSyn-C-trm-IRES-eGFP viral construct when compared to KO animals (Fig. 6.3a, p=0.0856) suggesting that WT Purkinje cells are transduced by the viral particles more easily or the Purkinje cells from KO mice have died regardless of viral expression. It was, however, found that the viral spread (measured as total thickness of the cerebellar tissue in which transduced Purkinje cells were found) showed no significant difference (Fig. 6.3b) between WT and KO animals demonstrating that the viral particles are able to diffuse through the cerebellar tissue in WT and KO mice in a similar fashion. Yet, when the viral spread was analysed taking the cerebellar region into consideration, it was found that in the anterior cerebellum of WT mice viral particles diffused much further than in posterior cerebellum (Fig. 6.3c, p=0.04225). These regional differences in viral diffusion were not observed in the KO mice (Fig. 6.3d). There was also a trend for lower transduced cell density (the ratio of total number of cells transduced and their spread) in KO mice when compared to WT animals (Fig. 6.3e, p=0.0803). Furthermore, it was also observed that in WT mice a significantly bigger percentage of the total transduced Purkinje cells were confined to the anterior cerebellar regions (Fig. 6.3f). In turn, a bigger proportion of transduced Purkinje cells in KO mice localised in posterior cerebellum.

When the number of virally transduced cells was correlated against their spread around the injection site, it was evident that in anterior regions of WT mice the slope was slightly gentler (Fig. 6.4a) when compared to posterior cerebellar regions in the same mice (Fig. 6.4b, p=0.2208). However, the slope in the anterior cerebellum
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Figure 6.2: Low-titre AAV1/2-eSyn-C-trm-IRES-eGFP construct shown to still express in anterior (top row) and posterior (bottom row) cerebellum of WT (left column) and KO (right column) mice 5 months after injection. White arrows point towards less visible Purkinje cells. Scale bar, 250 µm.

of WT mice differed significantly from the transduction slopes in anterior (Fig. 6.4c, p=0.002552) and posterior (Fig. 6.4d, p=0.04225) cerebellar regions of KO mice. No differences were detected in the transduction slopes between WT posterior, KO anterior and KO posterior cerebellum. These results suggest that in WT anterior cerebellar regions the viral particles are able to spread more easily and have a higher chance of successful cell transduction. The reason for this regional difference is not known but if
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Figure 6.3: AAV1/2-eSyn-C-trm-ires-eGFP transduced Purkinje cells in KO mice localise in posterior cerebellum. 
a) Total number of Purkinje cells transduced was similar in WT and KO mice, but was found to be slightly smaller in KO animals 
b) The spread of Purkinje cells transduced was comparable in WT and KO mice 
c) The spread of virally transduced cells was significantly lower in posterior cerebellum of WT mice (*p=0.0439, Student’s t-test) 
d) No differences in virally transduced cell spread was found in KO animals 
e) Cell density of transduced cells was slightly higher in WT animals 
f) Percentage of transduced cells localizing to the anterior cerebellum is significantly larger in WT mice, while bigger percentage of transduced cells in KO mice localise to the posterior cerebellar regions (*p=0.0458, Two-way ANOVA, N=5-8).
there is a cellular basis then there could be implications when considering viral delivery as a therapeutic strategy for cerebellar disorders.

**Figure 6.4:** Purkinje cells in anterior cerebellum of WT mice are transduced more efficiently. a), b) Correlation of virally transduced cells and their spread illustrated in anterior (a) and posterior (b) cerebellar regions of WT mice show different slopes of viral transduction c), d) Correlation illustrated in anterior (c) and posterior (d) regions of KO mice show similar transduction slopes across whole cerebellum (N=5-8).
6.2.3 High-titre AAV1/2-eSyn-C-trm-IRES-eGFP viral construct 9-month long expression has no effect on Purkinje cell spontaneous firing

A second attempt of viral production yielded significantly higher viral titre with much stronger viral transduction (Fig. 6.5a and Fig. 6.6). As gene therapy relies on stable protein product expression for prolonged periods of time, mice were injected at 3 months of age and sacrificed at 12 months of age allowing 9 months of viral expression.

Purkinje cells from β-III spectrin KO mice have been shown to spontaneously fire at lower frequency than Purkinje cells from WT mice at 6 weeks and 6 months of age (Perkins et al., 2010). Therefore to elicit a positive effect on motor performance, it would be predicted that expression of C-terminus (C-trm) β-III spectrin needs to enhance Purkinje cell activity. When the electrophysiological properties of Purkinje cells from transduced animals were evaluated following control injection of AAV1/2 CβAG-GFP viral particles, no effect was observed on Purkinje cell spontaneous firing (Fig. 6.5b). N=1 in anterior cerebellum injected with CβAG-GFP control AAV in Fig. 6.5b was due to lack of GFP labeled cells in anterior regions of the cerebellum following the injection. In contrast, after AAV1/2-eSyn-C-trm-IRES-eGFP injection into WT animals, virally transduced cells in the posterior cerebellum were firing at significantly lower frequency (Fig. 6.5c) suggesting viral toxicity. Although the AAV gene delivery system is considered non-toxic, significant cell toxicity has been shown in neuronal culture studies using AAV1 serotypes with functional GFP accentuating cell death (Howard et al., 2008). In KO mice, however, no effect was observed in either anterior, or posterior cerebellum (Fig. 6.5d). Similarly, in KO mice viral transduction did not have any effect on Purkinje cell firing modes in any of investigated cerebellar regions (Fig. 6.5e and f).
Figure 6.5: 9-month long AAV1/2-eSyn-C-trm-IRES-eGFP viral construct expression reduces spontaneous Purkinje cell firing in posterior cerebellum of WT mice. a) Confocal image of AAV1/2-eSyn-C-trm-IRES-eGFP expression 9 months after injection. Scale bar, 500 µm b) Control AAV1/2 CβAG-GFP expression for 9 months in WT mice has no effect on Purkinje cell firing (N=1-3, n=2-12) c) Spontaneous firing activity in posterior cerebellum of WT mice was significantly reduced after 9 months of C-trm β-III spectrin viral construct expression (*p=0.0211, Student’s t-test, N=3-4, n=10-18) d) No effect on Purkinje cell firing frequency in KO mice observed after the injection (N=3, n=7-13) e) No shift in Purkinje cell firing mode observed in either anterior (e, N=3, n=9-12), or posterior (f, N=3, n=10-13) cerebellum of KO mice.
Figure 6.6: High magnification of AAV1/2-eSyn-C-trm-IRES-eGFP transduced cells in WT (top section) and KO (bottom section) mice in sagittal cerebellar slices. Virally transduced cells are shown in first column (green), rabbit anti-ITPR staining of Purkinje cells in second column (red) and their overlay in the third column. AAV1/2-eSyn-C-trm-IRES-eGFP viral construct was consistently expressed mostly in Purkinje cells. Scale bar, 50 µm.
6.2.4 High-titre 2-week long AAV1/2-eSyn-C-trm-IRES-eGFP construct expression has no effect on Purkinje cell spontaneous firing

After evaluation of long term-expression of AAV1/2-eSyn-C-trm-IRES-eGFP viral construct, viral batch No.3 was produced. This viral batch was found to have a similar viral titre to batch No.2, therefore 3 month old mice were injected and electrophysiological properties were investigated 2 weeks later to check for any short-term viral injection effects. It was observed that the viral expression of C-trm $\beta$-III spectrin did not alter the spontaneous firing frequency either in WT, or in KO mice (Fig. 6.7). However, the transduced cell population in the posterior cerebellum of KO mice was found to have significantly higher variance when compared to non-transduced cells in the same region (Fig. 6.7b, $p=0.0112$).

![Figure 6.7](image)

**Figure 6.7:** 2-week long viral expression of AAV1/2-eSyn-C-trm-IRES-eGFP viral construct in Purkinje cells has no effect on spontaneous tonic firing. a) Purkinje cells in WT mice expressing the viral construct fire at the same frequency as non-transduced cells in the anterior and posterior cerebellum ($N=6-7$, $n=14-34$) b) Non-transduced Purkinje cells in KO mice anterior cerebellum fire significantly faster than non-transduced Purkinje cells from posterior regions, but the viral injection does not alter the firing frequency in KO mice (*$p=0.0262$, Two-way ANOVA, $N=4-5$, $n=14-18$).

During electrophysiological recordings it was noted that eGFP marker strength varied substantially from cell to cell. To investigate whether the strength of eGFP expression and hence C-trm $\beta$-III spectrin expression had an effect on Purkinje cell basal activity, an arbitrary score was given to each transduced cell ranging from 1 (hardly green, only visible with a corner of the eye) to 5 (bright green). These GFP scores
were correlated with tonic firing frequency for each cell. No significant correlation was observed in either anterior (Fig. 6.8a), or posterior (Fig. 6.8b) cerebellum of KO mice. The results illustrate that regardless of the eGFP expression strength, 2-week long AAV1/2-eSyn-C-trm-IRES-eGFP viral construct expression did not have any effect on Purkinje cell basal activity. Table 6.1 summarises injections performed and their effect on Purkinje cell spontaneous firing frequency.

Spontaneous cell firing after transduction

<table>
<thead>
<tr>
<th>Injection</th>
<th>WT Anterior</th>
<th>WT Posterior</th>
<th>KO Anterior</th>
<th>KO Posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk C-trm β-III spectrin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 mo C-trm β-III spectrin</td>
<td>-</td>
<td>⇓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 mo CβAG-GFP</td>
<td>-</td>
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</tr>
</tbody>
</table>

Table 6.1: Summary of injections performed and their effects on Purkinje cell spontaneous firing. Wk (week), mo (month), decrease (⇓) and no effect (-).

Analysis of Purkinje cell firing modes in the anterior (Fig. 6.8c) cerebellum of KO mice revealed that the viral expression did not alter the previously observed firing mode ratios (Fig. 3.5e) and there were no differences between non-transduced and transduced Purkinje cells. In the posterior cerebellum, however, there was a small shift in the firing mode as following the viral transduction, all Purkinje cells recorded from were firing (Fig. 6.8d), while some non-transduced cells were silent. Statistical analysis, though, did not reveal this difference to be significant. Summary can be seen in Table 6.2

Firing mode after transduction

<table>
<thead>
<tr>
<th>Injection</th>
<th>KO Anterior</th>
<th>KO Posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk C-trm β-III spectrin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 mo C-trm β-III spectrin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 mo CβAG-GFP</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Table 6.2: Summary of injections performed and their effects on Purkinje cell firing mode. No effect (-).
Figure 6.8: 2-week long viral expression of AAV1/2-eSyn-C-trm-IRES-eGFP viral construct does not alter Purkinje cell firing mode in KO mice. a), b) No correlation between eGFP expression intensity and Purkinje cell spontaneous firing frequency observed in either anterior (N=3-4, n=17-18) (a), or posterior (b) cerebellum of β-III spectrin spectrin KO mice c), d) No changes in the Purkinje cell firing mode seen in KO mice after delivery of C-trm β-III spectrin viral construct into anterior (c, N=3, n=15-18) and posterior (d, N=3-4, n=18-21) cerebellar regions.
6.2.5 Effects of short- and long-term expression of AAV1/2-eSyn-C-trm-IRES-eGFP on passive Purkinje cell membrane properties

In order to evaluate the passive properties of Purkinje cell membrane following a 2-week long AAV1/2-eSyn-C-trm-IRES-eGFP expression, a current injection protocol was followed (Fig. 6.9a). No changes in cell excitability (Fig. 6.9b) or input resistance (Fig. 6.9c) were detected in the anterior cerebellum of KO mice after viral transduction. In contrast, Purkinje cells from the posterior cerebellar regions showed increased cell excitability after stereotaxic viral injection (Fig. 6.9d). Furthermore, the input resistance was found to be significantly reduced (Fig. 6.9e), which would indicate bringing the KO Purkinje cells to WT level, as in the posterior regions KO Purkinje cells have higher input resistance (Fig. 6.14b and Fig. 3.8a).

Following 9-month long AAV1/2-eSyn-C-trm-IRES-eGFP expression, no differences in input/output relationship were observed, when transduced and non-transduced cells were compared in KO mice anterior (Fig. 6.10a) and posterior cerebellum (Fig. 6.10c). These results potentially suggest that the viral transduction effect could be ameliorated after long-term expression, indicating viral product instability. No effect on input resistance was detected in either anterior (Fig. 6.10b) or posterior cerebellum (Fig. 6.10d).

Evaluating 9-month long CβAG-GFP control expression showed no changes in cell excitability (Fig. 6.11a), nor input resistance (Fig. 6.11b). Theses results suggest that Purkinje cell viral transduction with AAV1/2-eSyn-C-trm-IRES-eGFP viral construct elicits no long-term effect on passive membrane properties. The summary of transduction effect on Purkinje cells in KO mice can be found in Table 6.3.

<table>
<thead>
<tr>
<th>Injection</th>
<th>Anterior I/O</th>
<th>Anterior IR</th>
<th>Posterior I/O</th>
<th>Posterior IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk C-trm β-III spectrin</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>9 mo C-trm β-III spectrin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 mo CβAG-GFP</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.3: Transduction effect on input/output (I/O) and input resistance (IR) in KO mice Purkinje cells. Increase (↑), decrease (↓), no effect (-), not applicable (NA).
Figure 6.9: 2-week long expression of AAV1/2-eSyn-C-trm-IRES-eGFP viral construct increases Purkinje cell excitability in posterior cerebellum of KO mice. a) Current injection protocol followed to produce input/output plots b), c) Purkinje cells from the anterior regions of KO mice show no differences in either cell excitability (b), or input resistance (c) after viral injection (N=5, n=12-18) d), e) Virally transduced Purkinje cells in the posterior cerebellum of KO mice show significantly increased excitability (d, p=0.0014-0.0188, Two-way ANOVA) and significantly reduced input resistance (e, *p=0.0139, Student’s t-test, N=5, n=15).
Figure 6.10: 9-month long AAV1/2-eSyn-C-trm-IRES-eGFP viral construct expression has no long-term effect on cell excitability in KO mice. a), b) No changes in cell excitability (a), nor input resistance (b) were observed in the anterior cerebellum of KO mice (N=4, n=8-13) c), d) No differences in cell excitability (c), or input resistance (d) were detected following the long-term viral expression in the posterior cerebellum of KO mice (N=4, n=11-16).

Figure 6.11: Long-term CβAG-GFP construct expression has no effect on either cell excitability (a), or input resistance (b) in posterior cerebellum of KO mice (N=2, n=7-8).
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Since the main goal of β-III spectrin delivery in KO mice Purkinje cells was to bring the cell’s electrophysiological and morphological properties to the WT Purkinje cell level, membrane properties of KO Purkinje cells were compared with non-transduced WT Purkinje cells, serving as healthy controls. It was observed that following 2-week long AAV1/2-eSyn-C-trm-IRES-eGFP expression transduced (Fig. 6.12c and d) and non-transduced (Fig. 6.12a and b) Purkinje cells from the anterior regions of KO mice cerebellum showed the same levels of cell excitability and similar input resistance as Purkinje cells in WT mice. In contrast, non-transduced and transduced Purkinje cells from the anterior cerebellum of injected 12-month old animals were significantly more excitable than Purkinje cells from age-matched WT mice (Fig. 6.13a and c, respectively). The input resistance remained comparable (Fig. 6.13b and d).

When the same comparisons were performed for the posterior cerebellum after the 2-week long viral expression, non-transduced KO Purkinje cells showed increase in cell excitability (Fig. 6.14a) as well as a significant increase in cell input resistance (Fig. 6.14b) as previously observed (Fig. 3.8a). Cell excitability (Fig. 6.14c) as well as cell input resistance (Fig. 6.14d) remained elevated after the viral transduction in KO mice when compared to WT Purkinje cells. As the difference in Fig. 6.14d was smaller than previously observed (Fig. 6.14b) and similar to non-injected age-matched animals (Fig. 3.8a), these findings might indicate that the viral transduction is bringing Purkinje cells from KO mice to WT Purkinje cell levels.

Analysis of 9-month long AAV1/2-eSyn-C-trm-IRES-eGFP expression suggests that although KO non-transduced Purkinje cells exhibit the same excitability as non-transduced WT Purkinje cells (Fig. 6.15a), the input resistance is significantly bigger (Fig. 6.15b). Increased cell input resistance was also found in the non-transduced Purkinje cells from the control CβAG-GFP injection animals (Fig. 6.15d), however, it was accompanied by increase in cell excitability as well (Fig. 6.15c). When the non-transduced Purkinje cells from WT mice posterior regions were compared to transduced Purkinje cells from C-trm β-III spectrin injection and control CβAG-GFP surgeries, cell excitability (Fig. 6.15e) as well as cell input resistance (Fig. 6.15f) were found to be significantly bigger. These
**Figure 6.12:** 2-week long expression of AAV1/2-eSyn-C-trm-IRES-eGFP in the anterior cerebellum has no effect on cell excitability. a), b) No difference in non-transduced Purkinje cell excitability (a), or cell input resistance (b) was observed between anterior cerebellar regions of WT and KO mice (N=5-6, n=13-18) c), d) In the anterior cerebellum, non-transduced Purkinje cells from WT mice exhibited similar cell excitability (c) and input resistance (d) as transduced Purkinje cells from KO mice anterior cerebellum (N=5-6, n=12-13).

Results illustrate that Purkinje cells from the posterior cerebellum are still significantly more excitable even following AAV1/2-eSyn-C-trm-IRES-eGFP expression. The summary of these findings can be seen in Table 6.4 and 6.5.
**Figure 6.13:** Cell excitability is not reduced following 9-month long C-trm β-III spectrin viral construct expression. a), b) Cell firing frequency following current-injection protocol is significantly increased (a) in non-transduced Purkinje cells in the anterior cerebellum of KO mice with no changes in input resistance (b) (p= 0.0073-0.0292, Two way ANOVA, N=3-4, n=8-9) c), d) Transduced cells are significantly more excitable (c) without any changes in their input resistance (d, p=0.0072-0.0379, Two-way ANOVA, N=3-4, n=11-13).

<table>
<thead>
<tr>
<th>Injection</th>
<th>Non-transduced KO vs WT</th>
<th>Transduced KO vs WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk C-trm β-III spectrin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 mo C-trm β-III spectrin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>9 mo CβAG-GFP</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Table 6.4:* Summary of injections performed on β-III KO mice anterior cerebellum and their effects on passive membrane properties when compared to non-transduced WT Purkinje cells. Increase (↑), no effect (-), not applicable (NA).
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Figure 6.14: Purkinje cell excitability remains elevated following 2-week long expression of AAV1/2-eSyn-C-trm-IRES-eGFP viral construct. a), b) Non-transduced Purkinje cells in the posterior cerebellum of KO mice showed significantly increased excitability (a, \(p=0.0122-0.0253\), Two way ANOVA) and input resistance (b) compared to non-transduced Purkinje cells from WT mice (****\(p<0.0001\), Student’s t-test, \(N=4-7, n=15-24\)) c), d) Transduced Purkinje cells from KO mice demonstrated elevated cell excitability (c, \(p=0.0202-0.0466\), Two-way ANOVA) as well as increased cell input resistance (d) when compared to non-transduced Purkinje cells from the posterior cerebellar regions of WT mice (**\(p=0.0029\), Student’s t-test, \(N=5-7, n=15-24\)).

Input/output and input resistance in posterior cerebellum after transduction

<table>
<thead>
<tr>
<th>Injection</th>
<th>Posterior</th>
<th>Non-transduced KO vs WT</th>
<th>Transduced KO vs WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk C-trm (\beta)-III spectrin</td>
<td>I/O ↑ ↑</td>
<td>I/O ↑ ↑ ↑ ↑ ↑</td>
<td></td>
</tr>
<tr>
<td>9 mo C-trm (\beta)-III spectrin</td>
<td>- ↑ ↑ ↑ ↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 mo C(\beta)AG-GFP</td>
<td>↑ ↑ ↑ ↑</td>
<td>↑ ↑ ↑ ↑</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.5: Summary of injections performed on \(\beta\)-III KO mice posterior cerebellum and their effects on passive membrane properties when compared to non-transduced WT Purkinje cells. Increase (↑), decrease (↓), no effect (-), not applicable (NA).
Figure 6.15: After 9-month long expression of C-trm β-III spectrin and CβAG-GFP elevated input resistance persists in Purkinje cells of posterior cerebellum. a) No differences in cell excitability between non-transduced WT and KO Purkinje cells from posterior cerebellum b) In the posterior cerebellum non-transduced KO Purkinje cells have significantly higher input resistance than WT Purkinje cells (*p=0.0208, Student’s t-test, N=3-4, n=12-16) c), d) Non-transduced Purkinje cells in the posterior cerebellum of CβAG-GFP injected mice have increased cell excitability (c, p=0.0149-0.0430, Two-way ANOVA) and significantly higher input resistance (d, *p=0.0349, Student’s t-test, N=2-3, n=7-13) e) Cell excitability after viral transduction with either viral construct is significantly higher when compared to WT non-transduced Purkinje cells (p=0.0076-0.0472, Two-way ANOVA) f) Significantly increased cell input resistance persists in the posterior cerebellum Purkinje cells following long-term cell viral transduction (**p=0.0003 and ***p=0.0004, One-way ANOVA, N=2-6, n=8-25).
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6.2.6 Cell morphology

The first batch of the virus had a low titre, and so not enough Purkinje cells were transduced for electrophysiological analysis. Nevertheless, it allowed sparsely labeled Purkinje cells to be analysed by confocal microscopy for morphological changes following the viral transduction. When individual Purkinje cells expressing the viral construct were imaged (Fig. 6.16) and analyzed (Fig. 6.17), it was found that Purkinje cells from the KO mice had significantly smaller dendritic tree areas in the anterior cerebellar regions when compared to the WT animals (Fig. 6.17a). Similarly, anterior cells from WT mice were also significantly larger than cells from the posterior regions in the same animals, suggesting that posterior and anterior Purkinje cells even under normal conditions are significantly different. Such a trend was also observed in Fig. 3.3a. Conversely, no significant difference was detected between WT and KO Purkinje cells dendritic tree surface area in the posterior cerebellum (a. These results indicate a possible modest effect of C-trm β-III spectrin on slowing dendritic degeneration.

No differences were found when the distance to the first dendrite (Fig. 6.17b) and to the dendritic tree (Fig. 6.17c) were compared between genotypes and cerebellar regions, whereas a significant difference was observed at this age in animals that have not undergone stereotaxic surgery (Fig. 3.4), further supporting modest positive effect of C-trm β-III spectrin viral construct after prolonged expression.
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Figure 6.16: Individual Purkinje cells from WT (left) and KO (right) mice transduced with AAV1/2-eSyn-C-trm-IRES-eGFP viral construct in the anterior (top row) and posterior (bottom row) cerebellum after 5 months of expression.
Figure 6.17: Viral β-III spectrin expression in KO Purkinje cells has a modest effect on dendritic tree morphology. a) Purkinje cells from KO mice expressing AAV1/2-eSyn-C-trm-IRES-eGFP viral construct have significantly smaller dendritic trees in the anterior cerebellum when compared to WT mice b) Quantification of distance to the first dendrite in Purkinje cells show no differences between the genotypes and cerebellar regions c) Quantification of distance to the dendritic tree in the virally transduced cells shows no difference between the genotypes and cerebellar regions (*p=0.0139-0.02, Two-way ANOVA, N=4-6, n=7-13).
6.2.7 Earlier AAV1/2-eSyn-C-trm-IRES-eGFP injection improves motor performance of β-III spectrin KO mice

During long-term viral expression of AAV1/2-eSyn-C-trm-IRES-eGFP and CβAG-GFP period, motor performance of injected mice was closely monitored. It was observed that KO mice expressing C-trm β-III spectrin and CβAG-GFP viral constructs were performing similarly in a rotarod test at 3 rpm, falling off significantly faster than WT mice (Fig. 6.18a). The WT mice injected with control GFP virus performed better than KO animals, but slightly worse than WT mice injected with β-III spectrin. This could have been due to small experimental groups, as some WT animals sometimes struggle with the rotarod task. Control surgery was performed earlier confirming that the surgery and short term GFP expression have no negative effects on mice motor performance (Fig. 6.1b).

In an elevated beam test (Fig. 6.18b) GFP and C-trm β-III spectrin expressing animals performed similarly. Both groups, however, made significantly more slips than WT control injection groups. Similarly, low titre AAV1/2-eSyn-C-trm-IRES-eGFP injection for 5 months yielded similar results with KO animals displaying deteriorating motor performance (Fig. 6.18c). No differences were detected either between the genotypes, or different injections when mice gait was analysed (Fig. 6.18d and e) following 9-month long expression of viral particles.

However, an earlier injection at 6 weeks of age was performed and resulted in a better performance on a rotarod (77.3±9.2s 6 months after the surgery) when compared with KO mice injected at 3 months of age with C-trm β-III spectrin (38.2±12.3 s, Fig. 6.19a). In an elevated beam test, a significant motor deterioration was observed (Fig. 6.19b), however the number of hind paw slips made was smaller (1.17±0.33 slips) when compared to 1.58±0.16 slips made by KO mice injected at 3 months of age with β-III spectrin. Of note, at 6 months after injection at 6 weeks of age, it was evident that motor performance was quite varied between β-III spectrin KO mice with only two mice making 2-3 times more hind paw slips (Fig. 6.19b). Time to cross the beam was not observed to change significantly (Fig. 6.19c). Additionally, no significant
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differences were detected in stride length (Fig. 6.19d) or width (Fig. 6.19e) with time. In summary, these results suggest that an earlier stereotaxic injection of AAV1/2-eSyn-C-trm-IRES-eGFP viral particles (at 6 weeks of age) may be beneficial at ameliorating motor deterioration observed in KO mice.

6.2.8 AAV1/2-eSyn-C-trm-IRES-eGFP viral particles hold potential as an effective gene therapy approach

This chapter reported some conflicting data following AAV1/2-eSyn-C-trm-IRES-eGFP viral particle delivery mice, however, a few findings indicate positive therapeutic effects. Firstly, following an initial β-III C-trm delivery it was determined that majority of transduced Purkinje cells in KO animals were found in the posterior cerebellar regions (Fig. 6.9e), the same region where majority of cellular degeneration was previously reported (Chapter 3).

It was previously presented in Fig. 3.8a that Purkinje cells from the posterior cerebellar regions of KO mice had a trend of elevated input resistance when compared to WT mice. Following a viral expression of AAV1/2-eSyn-C-trm-IRES-eGFP, transduced Purkinje cells had a significantly smaller input resistance in the posterior cerebellar regions of KO mice, indicating that AAV1/2-eSyn-C-trm-IRES-eGFP expression might bring KO Purkinje cell input resistance to WT Purkinje cell levels (Fig. 6.9e).

When Purkinje cell morphology was analysed following a 5-month long viral expression, it was observed that no significant difference was present between WT and KO mice posterior cerebellum Purkinje cell surface area (Fig. 6.17a), whereas a difference was previously detected at all ages tested in non-injected animals (Fig. 3.3d). Furthermore, no difference was observed between distance to the first dendrite in KO animals when anterior and posterior cerebellar regions were compared, in contrast to non-injected animals of the same age (Fig. 3.4b).

Finally, when animal motor abilities were tested in a rotarod test, it was observed that motor deterioration was present in animals injected at 3 months of age (Fig. 6.18), while the mice were much better off after viral particle delivery at 6 weeks of age.
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(Fig. 6.19) with mice not demonstrating the motor incoordination.
Figure 6.18: 9-month long expression of high-titre AAV1/2-eSyn-C-trm-IRES-eGFP viral construct in Purkinje cells does not prevent motor deterioration in KO mice. a) Rotarod performance at 3 rpm evaluated from 1 month (1ML) up to 9 months (9ML) after the surgery in WT and KO mice injected with either AAV1/2-eSyn-C-trm-IRES-eGFP (C-trm) or CβAG-GFP (GFP) viral constructs. No difference was observed between KO mice injection groups. C-trm injected WT mice performed significantly better than all KO mice, while GFP injected WT mice were performing only slightly better than KO mice (N=3) b) Summary of elevated beam test shows no difference between C-trm and GFP injection within WT and KO mice groups (N=3) c) Elevated beam test following 5-month long low-titre β-III spectrin injection shows significant deterioration of motor performance in KO animals (**p=0.0070, ****p<0.0001, Two-way ANOVA, N=5-8) d), e) No differences between WT and KO animals regardless of viral construct injection in gait length (d) and width (e) (N=3).
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Figure 6.19: Injection of high-titre AAV1/2-eSyn-C-trm-IRES-eGFP viral construct at 6 weeks of age significantly improves motor performance of KO mice on a spinning rotarod. a) Latency to fall off the moving rotarod at 3 rpm speed is significantly longer at 6 months after the surgery (*p=0.0348, One-way ANOVA) b) Hind-paw slips made while crossing the elevated beam significantly increased at 3 and 4 months after the surgery (*p=0.0383, **p=0.0023, One-way ANOVA) c) Time taken to cross the elevated beam remained the same before and for six months after the surgery d), e) No significant changes in stride length (d) or width (e) were observed for six months after the surgery (N=6).
6.3 Discussion

6.3.1 Control stereotaxic surgery has no effect on mice motor performance

In this chapter it was shown that stereotaxic surgery and overexpression of GFP has no significant effect on mice motor performance. Mice performance on the rotarod was completely unaffected at 3 and 5 rpm, with only a minor decrease in latency at 10 rpm. This deterioration, may simply be a consequence of the animals ageing, as WT animal motor performance does decrease in later life (Barreto et al., 2010). The fact that no other effects were observed in elevated beam test, stride or on animal weight provides strong supportive evidence that neither the procedure itself, nor overexpression of an exogenous protein has a negative effect on motor coordination short term.

6.3.2 Low titre AAV1/2-eSyn-C-trm-IRES-eGFP viral particles localise in posterior cerebellum of KO mice

In this section it was shown that a low-titre injection of AAV1/2-eSyn-C-trm-IRES-eGFP viral particles demonstrate a different expression profile in WT and KO mice. In WT animals, the same viral injection transduced more Purkinje cells than in KO animals, with most transduced cells localising in the anterior cerebellar regions. Furthermore, transduction slope in anterior regions of the WT mice was gentler than in KO animals, suggesting that not only more cells were transduced, but the viral spread was bigger in the anterior cerebellar regions of WT animals. In KO mice, however, most of the virally transduced cells localised in the posterior cerebellum. Taken together these results indicate that WT mouse cerebellum, especially anterior regions, are transduced more readily. More transduced cells might localise in the KO animal posterior regions due to the smaller volume of KO animal cerebellum. Reduction of the cerebellar volume is reported in SCA5 patients (Stevanin et al., 1999). In a β-III KO mice, the thinning of molecular layer has been observed at 6 months, but not 6 weeks of age (Perkins et al., 2010), with no published data for the 3 month time point. As
the coordinates of stereotaxic viral injection were kept the same between genotypes, more cells transduced in anterior cerebellum of WT mice would appear if the posterior cerebellum was not reached due to larger cerebellar volume. Also, as Purkinje cell degeneration may already be happening at 3 months of age, perhaps Purkinje cells are not as amenable to transduction and present at lower density. This ideally needs to be addressed in the future by staining all Purkinje cells and calculating the percentage of virally transduced cells 2 weeks and 9 months after the viral injection.

Furthermore, a viral application to cerebellar cultures would address whether WT and KO Purkinje cells transduce with similar ease. Understanding the reasons behind the dissimilarities in WT and KO mice viral transduction would allow to either improve the viral delivery techniques or could potentially reveal some cellular abnormalities playing a role in the SCA5 disease pathology.

6.3.3 High-titre long-term AAV1/2-eSyn-C-trm-IRES-eGFP viral particle expression has similar effect as control CβAG-GFP particles

It was shown that in the SCA5 mouse model, which lacks β-III spectrin, Purkinje cells are firing at significantly lower frequencies. The data in this chapter shows that neither C-trm β-III spectrin viral construct, nor CβAG-GFP viral particles had any immediate effect on spontaneous firing frequency of Purkinje cells. In a study performed by Dell’Orco et al. (2015), using the SCA1 mouse model established by Burright et al. (1995), the AAV therapeutic approach of introducing voltage- and Ca\(^{2+}\)- activated K\(^{+}\) (BK) channel was observed to significantly increase spontaneous firing in the transduced cells (Kasumu et al., 2012b; Dell’Orco et al., 2015).

In our study, the long term expression of AAV1/2-eSyn-C-trm-IRES-eGFP, however, reduced firing frequency in the posterior cerebellum of WT mice, illustrating a potentially neurotoxic effect. Neurotoxicity due to AAV system and GFP overexpression has been previously demonstrated (Howard et al., 2008). This effect, however, could be due to small experimental groups, as no effect of prolonged CβAG-GFP par-
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ticle expression was observed in this group. Of note, the CβAG promoter does not express strongly in Purkinje cells. Therefore, in the future eGFP should be engineered to be expressed from the same promoter (eSyn) as the C-trm β-III spectrin.

Results from the current injection protocol showed no difference in cell excitability or input resistance between non-transduced and transduced Purkinje cells following long term expression of AAV1/2-eSyn-C-trm-IRES-eGFP and CβAG-GFP in KO mice. In contrast, 2-week long viral expression of C-trm β-III spectrin in the posterior cerebellum of KO mice significantly increased cell excitability while reducing input resistance. These results demonstrate that the effect of viral transduction could be lost with prolonged periods of viral expression and, in order to maintain them, repeated viral expression might be required.

The issue of repeated viral administration has been addressed in multiple publications. In other organ systems rather than the brain, an immune reaction is sometimes observed reducing the viral product longevity in liver and lungs (Daya and Berns, 2008). Repeated AAV1, AAV2 and AAV5 introduction has been shown to have 10 to 100 times lower AAV expression levels when compared to the first viral injection in mice skeletal muscle (Riviere et al., 2006) and increased levels of neutralising antibodies were detected following a repeated administration of AAV2 in retina (Barker et al., 2009). A study by Löw et al. (2013) demonstrated that in the rat brain after an AAV6 injection, a repeated delivery of AAV is twice as efficient when a mixture of AAV6 and AAV9 vectors rather than just AAV6 is administered. This could be explained by the hypothesis that the same AAV type is not able to transduce antigen expressing cells due to the previous transduction, suggesting that some immune reaction is observed in the brain (Zaiss and Muruve, 2005). Mastakov et al. (2002) also addressed this issue in the brain and found that the repeated viral injection produced lower protein expression if done within two weeks of the first administration. If the second injection is performed 4 weeks or later, no significant decrease in viral expression levels was observed, suggesting that the timing of the second injection is a crucial variable to be considered when planning the repeated AAV delivery experiment.
Reduction of the input resistance in the posterior cerebellum following the 2-week long expression is a promising finding, as Purkinje cells in non-injected KO animals have an elevated input resistance (Chapter 3). As input resistance is inversely proportional to the cell surface area, reduction in input resistance might indicate an increased cell surface area. As observed in Chapter 3, KO Purkinje cells in the posterior cerebellum have significantly decreased dendritic trees, therefore reduction in input resistance difference between WT and KO cells in the posterior cerebellum might be an indication of an increasing dendritic tree. Alternatively, reduction in the input resistance might also indicate changing properties of the cell membrane, such as an increase in amount of leak current channels. A study by Dell’Orco et al. (2015) applied flufenamic acid (FFA) on cerebellar slices from 5-week old SCA1 mice (Burright et al., 1995) and found a significant decrease in input resistance. FFA activates BK and subthreshold potassium channels (Ottolia and Toro, 1994; Takahira et al., 2005). Systemic application of FFA for 6 weeks after inserting a FFA-delivering pump into a mouse was found to prevent Purkinje cell dendritic tree degeneration (Dell’Orco et al., 2015). These results demonstrate that input resistance and dendritic tree are tightly related and that decrease in input resistance might indicate improved dendritic tree morphology.

6.3.4 Long-term AAV1/2-eSyn-C-trm-IRES-eGFP viral particle expression has modest effect on KO Purkinje cell morphology

Unfortunately, the surface area of the Purkinje cell dendritic tree from KO mice expressing C-trm β-III spectrin in the anterior cerebellum is significantly smaller when compared to WT mice, while in the posterior cerebellum the dendritic trees are comparable in WT and KO mice, eliminating previously observed difference (Fig. 3.3). Similarly, the distance to the primary dendrite is comparable between the genotypes in the anterior and posterior cerebellum, although it was significant between non-injected KO and WT mice (Chapter 3). These results illustrate that the long-term expression of C-trm β-III spectrin may have a protective role on KO mice Purkinje cell dendritic tree. These results are similar to the study performed by Lin et al. (2015), where Purk-
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inje cell degeneration was prevented by delivery of GALK enzyme, which is missing in
globoid cell leukodystrophy.

In this study viral delivery of C-trm β-III spectrin did not have any significant effect
on Purkinje cell spontaneous firing. As Purkinje cells are the sole cerebellar output,
their normal activity is expected to be required for improving the ataxic phenotype.
Viral transduction was observed to marginally improve cell dendritic tree morphology
and some reduction in input resistance was also observed. It may be that these changes
were too mild to have an effect on Purkinje cell firing.

6.3.5 Earlier injection of AAV1/2-eSyn-C-trm-IRES-eGFP viral par-
ticles is more beneficial at preventing motor performance dete-
rioration

In Fig. 6.18 data showed that long-term high-titre expression of AAV1/2-eSyn-C-trm-
IRES-eGFP viral particles have similar effects as CβAG-GFP viral injection. Similarly,
a low-titre injection does not seem to prevent motor deterioration in β-III spectrin
KO mice. As all of these injections were performed at around 3 months of age, it
was hypothesised that the β-III spectrin delivery might be too late. In other studies
of cerebellar neurodegenerative conditions, improvements in motor performance were
observed after injecting the animals much earlier, at P3 (Lin et al., 2015) or at 7 weeks
of age (Xia et al., 2004). Injection of viral particles into 6 week old KO mice, indeed
resulted in a better performance during rotarod test (Fig. 6.19) when compared to
previously published observations (Perkins et al., 2010); at 6 months of age (Fig. 6.19a),
4 months after the viral injection, the mice were still lasting 57.2±9.9 s on a rotarod
while the age matched animals injected at 3 months of age only lasted 26.20±4.3 s
(similar to (Perkins et al., 2010)), suggesting some motor improvement.

Although injected mice did make more slips on the elevated beam, the number of
slips made stayed around the same 6 months later, suggesting slower motor deteriora-
tion. The increased variability between different mice at 6 months post-injection was
also evident. This could indicate either a varied response to the viral expression or
perhaps differences between the surgeries: if some stereotaxic injections were inaccurate, mice might be potentially performing worse in motor function evaluation tests. Furthermore, some variation in the number of Purkinje cells transduced was observed following 9 months of viral expression. Perhaps the number of Purkinje cells transduced correlates with the improvement in motor physiology. To reduce this variation of viral delivery, a study could be proposed where the viral delivery would occur in P0 or P1 with viral injection targeting cerebral ventricles. Injection at such early stage would allow the virus to cross the blood-brain barrier and, depending on AAV capsid type as well as timing of the injection, provide a strong transduction of cerebellar Purkinje cells (Zhang et al., 2011; Chakrabarty et al., 2013).

Viral delivery in this study was performed at 6 weeks and 3 months of age. No behavioural improvement was seen after injection at 3 months of age, with some promising results following the injection at 6 weeks of age. If we consider 6 week old mice to be equivalent to approximately 17 years of age in humans (and 3 months of mice age to 23 years of human age) such therapeutic interventions would not be possible, as patients are usually diagnosed in their thirties or later. By that time, the molecular changes underlying the symptoms had already occurred with little chance of Purkinje cell morphology and physiology reversal. Genetic testing within families with SCA5 would appear to be needed to guarantee a timely intervention to prevent the progression of disease pathology.
Chapter 7

General discussion

7.1 Summary of findings

Findings presented in this thesis show regional selectivity in a mouse model of spinocerebellar ataxia type 5 (SCA5) with Purkinje cells in the posterior cerebellum most susceptible to dendritic tree degeneration and cell death. These Purkinje cells were also observed to be significantly more excitable and possess higher input resistance than Purkinje cells from the posterior cerebellum of wild type (WT) mice. Work presented in the thesis also reveals that morphological abnormalities of β-III spectrin knock-out (KO) cerebellar Purkinje cells can be recapitulated in primary Purkinje cell cultures, with KO Purkinje cells exhibiting significantly more dendritic branching points, but possessing a smaller surface area than Purkinje cells from WT mice. Furthermore this pathophysiology was confirmed to be cell-autonomous, as plating WT and KO Purkinje cells together did not influence the morphology of the other genotype. Data in this thesis also demonstrated that primary cerebellar cultures are an excellent tool for manipulating dendritic tree formation pathways, providing a very quick readout. Using this protocol it was observed that T-type calcium channels play an important role in Purkinje cell dendritic tree formation and that their chronic block may prevent KO Purkinje cells from forming multiple dendrites. Finally, to address the reversibility of ataxia, C-terminus (C-trm) of β-III spectrin was identified to have comparable phys-
iological effects to that of full length (FL) β-III spectrin, both in Human Embryonic Kidney (HEK293FT) cells and hippocampal pyramidal neuron cultures. HEK293FT transfection and immunohistochemistry experiments identified C-trm β-III spectrin as the only fragment localising at the membrane where FL β-III spectrin is usually found. Furthermore, C-trm did not exert any dominant negative effect on FL β-III spectrin localisation. Electrophysiological recordings from the hippocampal neuronal cultures transfected with C-trm β-III spectrin showed increased sodium currents which were indistinguishable from the effects observed following transfection with FL β-III spectrin. However, the viral construct expressing C-trm β-III spectrin had only a modest effect alleviating SCA5 pathophysiology. Injections performed at 3 months of age did not appear to have any positive effect on the motor performance of KO mice, however, earlier injections (at 1.5 months of age) yielded promising preliminary results by slowing down the disease progression. Electrophysiological recordings have also revealed elevated cell excitability in transduced Purkinje cells following both, long term and short term viral transduction.

It would therefore appear that injection as early as possible is required in order to elicit any positive physiological effect on β-III spectrin KO mice. Furthermore, comparison of transduced KO mice Purkinje cells to non-transduced WT Purkinje cells, revealed that abnormally high cell excitability in KO mice persists following both, long-term and short term viral transduction, suggesting that the viral therapy did not bring the KO Purkinje cells to WT cell levels and a different approach might be needed for future experiments to fully alleviate the disease phenotype.

7.1.1 Future directions of the work

As Purkinje cells from the posterior cerebellum of KO mice were observed to be more excitable with a trend for higher input resistance in (Fig. 3.8), future experiments should address whether ion channel expression profile differs across different cerebellar regions. For example, distribution of sodium channels responsible for resurgent currents could be investigated in different cerebellar regions (Na\textsubscript{V}1.1 or Na\textsubscript{V}1.6). Furthermore, a
KO mouse line which lacks the Excitatory Amino Acid Transporter 4 (EAAT4), should be examined to address whether regional differences as well as cell morphological and physiological heterogeneity are present in EAAT4 KO mice.

As KN62 application had a drastic effect on WT cerebellar Purkinje cell cultures (Fig. 4.9 - Fig. 4.12), future experiments should target different calcium signaling pathways such as phospholipase C (PLC). Alternatively, calcium stabilising drugs such as ceftriaxone (Maltecca et al., 2015) or dantrolence (Chen et al., 2008) could be administered to β-III spectrin KO mice at various ages to investigate whether reducing calcium signaling alleviates motor incoordination symptoms as well as affect cell morphological and physiological properties.

7.2 Outstanding questions

7.2.1 Purkinje cell degeneration

What are the mechanisms behind regional cell degeneration?

This study showed that Purkinje cells from the posterior cerebellum of KO mice are more vulnerable to neurodegeneration when compared to the anterior cerebellar regions. Electrophysiological and immunohistochemistry experiments demonstrated that Purkinje cells from the posterior cerebellum have significantly smaller dendritic trees, fire at much lower frequencies and die first when compared to anterior cerebellum. Such region specific degeneration is also observed in other SCAs, but the underlying mechanisms of it is not yet clear. For example, in an SCA6 mouse model, which lacks P/Q voltage-gated calcium channels (VGCCs), the Purkinje cells most affected are in the anterior cerebellar regions, which have low EAAT4 expression (Fletcher et al., 2001).

Experiments presented in this thesis using primary cerebellar cultures have also revealed that the Purkinje cell abnormalities observed in KO mice are cell-autonomous. Similar observations have also been made in other models of ataxia, such as SCA1 (Burright et al., 1995) and Niemann-Pick disease (Elrick et al., 2010).

As previously discussed in Chapter 3, heterogeneity in the cerebellum could be ex-
explained by the fact that some proteins in the cerebellum are expressed in a regional manner. EAAT4, which is stabilized by β-III spectrin (Jackson et al., 2001), is normally expressed more strongly in the posterior cerebellum (Wadich and Jahr, 2005) and regulates metabotropic glutamate receptor (mGluR) activation (Brasnjo and Otis, 2001). Although an increased glutamate release has been demonstrated in EAAT4 rich regions (Paukert et al., 2010), it normally leads to little mGluR activation in high EAAT4 level expression regions (posterior cerebellum) (Wadich and Jahr, 2005). As a result, a normal balance between glutamate concentration and signaling is established in WT animals (Fig. 7.1, left). As EAAT4 shows reduced stability in the absence of β-III spectrin (Perkins et al., 2010), lack of EAAT4 at these synapses of the posterior cerebellar regions might lead to increased glutamate signaling (Fig. 7.1). This could then lead to increased excitation of Purkinje cells in the posterior cerebellar regions through AMPA and mGluR receptors, eventually causing cell degeneration. Dark cell degeneration, which is associated with excessive glutamate signaling (Garthwaite and Garthwaite, 1991), has also been observed in SCA28 (Maltecca et al., 2009). Of note, EAAT4 KO mice have been reported to exhibit larger parallel fibre (PF) - mediated excitatory postsynaptic currents (EPSCs) when compared to WT mice, indicating disturbed glutamate signaling (Perkins et al., 2016b).

As glutamate signaling is observed to alter intracellular Ca\(^{2+}\) concentration (Finch and Augustine, 1998; Kitamura and Kano, 2013), the direct consequence of excessive glutamate receptor activation would be increased calcium signaling. Deranged calcium signaling has been implicated in the pathophysiology of SCA2 (Kasumu and Bezprozvanny, 2012a). Furthermore, Long et al. (2014) showed that mice, lacking calmodulin-binding transcription activator 1 (which plays a key role in calcium signaling), develop ataxic phenotype and undergo Purkinje cell degeneration. Dark cell degeneration has also been demonstrated to be linked to rises in intracellular calcium levels (Strahlendorf et al., 1998) and activation of calpain, calcium dependent-protease (Mansouri et al., 2007). Maltecca et al. (2015) showed that in SCA28 mouse model, attenuation in calcium levels is observed and ataxic phenotype alleviated when mGluR1
activity is reduced. Additionally, Ca\(^{2+}\)/calmodulin-dependent protein kinase II isoform \(\alpha\) (\(\alpha\)CaMKII) expression has been shown to vary throughout the cerebellum with strongest expression in the posterior (Wang et al., 2013). Therefore increased glutamate signaling in the posterior cerebellum due to EAAT4 loss could be hypothesised to increase calcium signaling and have detrimental effects on cell morphology and survival.

Figure 7.1: Purkinje cells in the posterior cerebellum of \(\beta\)-III spectrin KO mice have elevated glutamate and calcium signaling leading to morphological changes and cell death. Posterior cerebellar regions of WT mice (left) express high levels of EAAT4, which efficiently removes glutamate (gray spheres) from the synaptic cleft resulting in little activation of AMPA and mGluR receptors and, subsequently, low level calcium signaling. \(\text{Cav}3\) (T-type) channel activation occurs in WT mice Purkinje cells around resting membrane potentials. In the posterior regions of \(\beta\)-III spectrin KO mice (right), the absence of \(\beta\)-III spectrin (red lines), destabilises EAAT4 at the membrane, significantly increasing glutamate concentration in the synaptic cleft. This in turn results in excessive AMPA and mGluR receptor stimulation and elevated calcium signaling in the cell. Due to AMPA activation and cellular depolarisation, T-type calcium channels are also hypothesised to activate earlier. Elevated calcium levels and excessive AMPA receptor activation are then expected to lead to dendritic tree changes and dark cell degeneration, respectively. Image adapted from Perkins et al. (2016a)

Future experiments, therefore would need to address whether \(\alpha\)CaMKII expression profile is disturbed in \(\beta\)-III spectrin mice and whether \(\alpha\)CaMKII levels differ across cerebellar regions of KO mice. Organotypic cerebellar cultures of anterior and posterior cerebellum could also be used. Such a system would allow evaluation of the developmental process of anterior and posterior cerebellum and independent manipulation of separate cerebellar regions through drug application.
What is the role of mGluRs in Purkinje cell degeneration?

Purkinje cell innervation by multiple climbing fibres was reported in mGluR mutant mice (Alba et al., 1994). Increased glutamate signaling is proposed to occur in β-III spectrin KO mice as discussed in Section 7.2.1 and mGluR1 activation increases calcium signaling through PLC signaling pathways (Netzeband et al., 1997). Experiments in Chapter 4 showed that CaMKII blocker KN62 has a very potent effect on Purkinje cell dendritic tree morphology. Perhaps in the future experiments drugs modifying PLC pathway (such as U73122 or edelfosine (Horowitz et al., 2005)) could be used to address what effect mGluRs overactivation plays in Purkinje cell dendritic tree abnormal morphology in KO mice. Innervation from multiple climbing fibres has also been observed in weaver mice (Crepel et al., 1976), reeler mice (Mariani et al., 1977) and staggerer mice (Crepel et al., 1980). Future experiments should therefore address whether Purkinje cells in β-III spectrin KO mice receive abnormal innervation by multiple climbing fibres.

The connection between a cell’s electrical activity and its dendritic development has been shown in multiple studies (Katz and Shatz, 1996; McAllister, 2000). Findings reported in this thesis that cells from the posterior cerebellum exhibit degenerating dendritic trees, have higher input resistance and are hyperexcitable are in line with other studies with similar properties observed in leaner mouse model (Ovsepian and Friel, 2008) as well as SCA1 (Inoue et al., 2001). It has been proposed that degenerated dendrites and smaller cell body would potentially keep cells closer to the firing threshold (Inoue et al., 2001). Therefore prevention of dendritic tree degeneration could prevent abnormal cell output as well as preserve Purkinje cell synapses with climbing and parallel fibres.

7.2.2 Is partial protein delivery really enough to alleviate SCA5?

The main goal of the project presented in this thesis was to explore therapeutic strategies for SCA5 patients. Introduction of viral vectors in order to correct for malfunctioning genes in monogenic disorders is one of the three main categories which gene
therapy research is used for, the other two being targeting aberrant cells (for example, in cancer) and stimulating production of therapeutic protein (McCain, 2005). SCA5 is thought to be brought about by the dominant negative effect due to reduction in WT protein levels (Lise et al., 2012). In this study adeno-associated viruses (AAVs) were used for cloning C-trm of β-III spectrin and for its delivery into KO mice.

In pre-clinical AAV studies correcting the abnormal gene expression and returning the affected protein has been successful in Hemophilia B, in which coagulation factor IX has been delivered (Nathwani et al., 2011). The researchers observed, however, the transient nature of protein expression, which was thought to be caused by neutralising antibodies in the host targeting transduced hepatocytes (Manno et al., 2006). Additionally, some AAV safety concerns have also been raised as AAV transduction was found to increase tumorigenesis in rodent liver questioning the safety of AAV use in gene therapy (Donsante et al., 2001).

In the brain, however, viral transduction seems to be quite stable and in this study the reporter enhanced green fluorescent protein (eGFP) was observed to still be expressed 9 months after injection. Yet the effect of β-III spectrin transduction, however, was observed to be transient with cell input resistance decreasing 2 weeks after injection, but not 9 months after injection. The cell excitability was also found to be increased after 2 weeks but not 9 months after viral injection. In the future stability of the transgene should be addressed by analysing its levels of expression at different time points. What is more, repeated AAV injection should also be considered as it has been demonstrated to be a viable approach by Mastakov et al. (2002) if done 4 or more weeks apart.

AAV viral transduction has been previously observed to be stable in the brain, as discussed in Chapter 6, and therefore another possibility for the transient viral effect could be the effect of eGFP expression. GFP overexpression has been previously demonstrated to be toxic to neurons (Klein et al., 2006), therefore its long term expression in Purkinje cells could have contributed to little improvement seen in the dendritic tree morphology.
As Chapter 3 showed the selective vulnerability of posterior cerebellum, the viral delivery in this project targeted mostly posterior cerebellum. In future studies, however, wider spread of the virus should be attempted, by performing multiple injections, which has been shown to be a viable approach.

It was also observed that the KO mice showed promising signs of delayed motor dysfunction after early (6 weeks of age), rather than late (3 months of age) injection. These findings suggest that in order to be effective, gene delivery should be performed as early as possible. In order to achieve that in patients, perhaps genetic testing of newborns should be offered to families at high risk of SCA5.

Finally, it is also possible that the modest effect elicited by the C-trm β-III spectrin introduction into mice was observed due to the partial protein reintroduction which might not be enough to prevent SCA5. To address this, an alternative viral vector could be used which would be able to accommodate a protein of bigger size. Ideally, a conditional SCA5 mouse model should be produced. In this way, not only FL β-III spectrin would be delivered, but also its expression could be tightly controlled in order to investigate the latest time point for effective gene therapy in order to prevent progressive motor dysfunction.

More than 1700 clinical trials are being carried out worldwide in order to investigate efficiency of gene therapy in genetic conditions (Giacca and Zacchigna, 2012), although few viral therapies have been approved (Bender, 2016). Their safety and stability are still to be confirmed in patients, as once administered such therapy cannot be stopped (Bender, 2016). Furthermore, additional ways of delivering the viral particles to the patients suffering from brain abnormalities should be considered, as currently each patient would require a brain surgery for viral delivery and follow up surgeries for transgene boosts.

7.2.3 What is the cerebellum’s role in normal brain function?

Classical anatomy studies have shown that the cerebellum receives innervation from multiple cortical areas, information then goes through cerebellar circuits and finally
reaches the ventrolateral nucleus of thalamus (Allen and Tsukahara, 1974). In turn, thalamus projects to the primary motor cortex. As a result, up until recently the cerebellum has been thought of as a structure only contributing to movement execution (Strick et al., 2009). However, some researchers demonstrated that cerebellar lesions could also have psychiatric symptoms and cognitive impairment symptoms (Petersen et al., 1989; Kim et al., 1994; Schmahmann, 2004).

Anatomically it has been recognized that the cerebellum actually projects to multiple regions of the ventrolateral nucleus of thalamus (Percheron et al., 1996) which then innervates widespread cortical areas (Strick et al., 2009). Lateral hemispheres of the cerebellum, which have expanded the most during brain evolution have been found to project to ‘cognitive’ association cortices such as frontal and prefrontal cortices and posterior parietal cortex (Leiner et al., 1993; Stoodley, 2012). This suggests that cerebellar output could potentially affect more cortical areas than previously thought and therefore have additional roles which have not yet been established. Cerebellar activation has been observed in attention tasks, when more concentration was required (Allen and Courchesne, 2003), in verb generation tasks (Fiez et al., 1996) as well as during mental calculation (Hanakawa et al., 2003) and in Tower of London task which requires planning and working memory (Baker et al., 1996).

Cerebellar abnormalities have also been observed in autism (Ito, 2008). For example, a study by Carper and Courchesne (2000) showed cerebellar volume reduction in patients with autism. Furthermore, Purkinje cell abnormalities have also been observed in autism study by Fatemi et al. (2002) showing a 50% reduction in Purkinje cell size. In attention tasks autistic patients also demonstrate significantly lower activation of the cerebellum (Allen and Courchesne, 2003).

Most patients with heterozygous mutations in SCA5 only demonstrate signs of cerebellar dysfunction with no identified cognitive deficits (Stevanin et al., 1999; Bürk et al., 2004; Ikeda et al., 2006). A publication by Lise et al. (2012) showed that homozygous mutations in SPTBN2 gene also cause Spectrin-associated Autosomal Recessive Cerebellar Ataxia type 1 (SPARCA1), the patients of which demonstrate cognitive
impairment with poor IQ scores and delayed developmental profile with onset at childhood. Another homozygous mutation (c.2864_2868del 5 bp deletion) was also identified to have a global developmental delay with very early onset. Also, a heterozygous arginine to tryptophan substitution identified by Jacob et al. (2013) caused a more severe phenotype with global developmental delay.

In the collaborative study with Lise et al. (2012) the cognitive function of β-III spectrin KO mice was evaluated. It was observed that although KO mice performed just as well as WT mice in 2 novel object recognition task, they showed some impairment in a 4 novel object recognition task. Furthermore, they were significantly worse at identifying swapped objects in object-in-place-task. These findings suggest that β-III spectrin has an important role in normal brain function. Future experiments therefore should address what role β-III spectrin plays in normal brain function. Additionally, it should be investigated whether any autistic patients have abnormalities in β-III spectrin expression.

7.2.4 Final summary

The work in this thesis has demonstrated selective regional degeneration of Purkinje cells in a SCA5 mouse model, signs of which are dendritic degeneration, increased input resistance and increased cellular excitability. It was also shown that Purkinje cerebellar cultures can be used as a quick readout in drug studies in order to investigate pathways involved in dendritic tree formation. It was also demonstrated that a partial protein introduction by viral therapy holds some promise, however the genetic intervention should occur as early as possible. Finally, the experiments outlined here suggest that the effect of C-trm β-III spectrin expression was transient and therefore for any AAV gene therapy follow-up injections will likely be necessary.
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Appendices
Appendix A

MATLAB Scripts

A.1 Surface area calculation

```matlab
[filename, path] = uigetfile({'*.tif'}, 'Select file(s)');

file = sprintf('%s%s', path, filename);
img = imread(file);
imginfo = imfinfo(file);

resolution = 1 / imginfo.XResolution;
stem_sensitivity = 0.7; % higher is more sensitive, must be under 1.
brightness = 0.20;

imgbw = im2bw(img, brightness);
figure(1);
image(img);

comps = bwconncomp(imgbw);
sizes = cellfun(@numel, comps.PixelIdxList);
[size, idx] = max(sizes);

imgbw(1:comps.ImageSize(1), 1:comps.ImageSize(2)) = 0;
imgbw(comps.PixelIdxList{idx}) = 1;
```
figure(2);
image(imgbw*10);
cellsizex=size*(resolution^2);
sprintf('Size of the cell (um^2): %f', cellsizex)

imgbwdark = im2bw(img, 0.5);
erode = floor(sqrt(sum(sum(imgbwdark))) / 8);
ingbwdark = imerode(imgbwdark, strel('disk', erode));
comps = bwconncomp(imgbwdark);
sizedark = cellfun(@numel, comps.PixelIdxList);
[sizedark, idx] = max(sizedark);

xs = mod(comps.PixelIdxList{idx}(1), comps.ImageSize(1));
ys = floor(comps.PixelIdxList{idx}(1)/comps.ImageSize(1));

found_stem = 0;
found_dendrite = 0;
i = 1;
last_count = 0;
while found_dendrite == 0
    count = 0;
    for x = max(xs-i, 1):min(xs+i, comps.ImageSize(1))
        for y = max(ys-i, 1):min(ys+i, comps.ImageSize(2))
            count = count + imgbw(x, y);
            imgbw(x, y) = 0;
        end
    end

    count = count / i;

    if found_stem == 0
        if last_count > count * 1.2
            found_stem = 1;
        end
    elseif last_count < count * stem_sensitivity
        found_dendrite = 1;
    end
end

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end

last_count = count;
i = i + 1;

if i > 300
    found_dendrite = 1;
end
end

hull = bwconvhull(imgbw);

sprintf('Size of spread (um^2): %f', sum(sum(hull))*(resolution^2));
figure(3);
image(xor(hull, imgbw));
A.2 Frequency analysis

A.2.1 Frequency analysis script

close all;

path = fileparts(mfilename('fullpath')); %mfilename takes the whole ...
       path, fileparts splits the name (firing single or joint) from the ...
       rest of the path

delete(sprintf('%s/Output/Frequency/*.xlsx', path));
addpath(sprintf('%s/Includes', path));

[filenames, path] = uigetfile({'* .abf'}, 'Select file(s)', ...
       'MultiSelect', 'on'); %filenames is a list of filenames I selected ... 
       in the dialog box

if ~iscell(filenames) %if filenames is not an array
       filenames = {filenames};%make it into one element array. we want it ... 
       in am array because you cannot have text inthe matrix
end

number_of_files = length(filenames); %length is a function getting a ...
       number

m = 1;

for i = 1: number_of_files
       fullname = strcat(path, filenames{i});%strcat concatenates 
       data = abfload(fullname{1});
       name = filenames{i};

       if isempty(data)
              continue %means skipping everything after this and get to the ... 
       next section in case there is something wrong with the data
       end

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duration = size(data, 1);
sweeps = size(data, 3);
sq = ceil(sqrt(sweeps));
data = reshape(data, duration * sweeps, 1);

figure(99);
plot(data(1:duration*sweeps, 1));
title(filenames(i));

filter = 10000;
total_length = floor(duration * sweeps / filter);
if total_length < 100
    filter = 0;
end

if filter == 0
    close 99;
    continue;
end

if total_length == 100
    startts = 50;
    endts = 100;
duration = (endts - startts) * filter;
fullname = sprintf('%s %d:%d', name{1}, startts, endts);
[ISI_values, AP_actual_sizes, ...
    AP_times_number] = Analysis_50s(data((startts * filter + ... 1):(endts * filter)), 1, m, 9, filter, fullname, ...
    'Frequency', path, filenames{1}, filenames(i), duration);
m = m + 1;
end
A.2.2 Frequency analysis function

function [ISI_values, AP_actual_sizes, AP_times_number] = Analysis_50s(data, sweep, k, k_total, filter, filename, ...
output_folder, location, name, file, duration)

% Function Analysis

% after '=' is the name of the function and in brackets there is a list of % arguments

% Arguments:
% matrix @data –
% int @sweep – which sweep I am analysing
% int @k – kelintas failas is visu failu yra nagrinejamas
% int @k_total – kiek failu is viso yra atidaroma vienu kartu
% int @filter (default: 10000) –
% string @filename (default: Data file) –
% string @output_folder (default: Default) –
% Returns (lauztiniai skliaustai salia function, which values the ... function will return)
% matrix ISI_values –
% matrix AP_sizes –
% int AP_number –

% arguments are taken from the file that function is called out from. The % ORDER of the arguments matters, not the names

formatOut = 'HH-MM-SS';
fulltime=strcat(date,{' '},datestr(now,formatOut));

if nargin < 5 % if less than 5 arguments, filter becomes default
filter = 10000;
if nargin < 6
    filename = 'Data file'; % just a default heading
end

if nargin < 7
    output_folder = 'Default'; % just a name, we do not use it anywhere
end

% these are necessary so I would not need to pass these arguments when I do
% not need to pass them

k_rows = ceil(sqrt(k_total)); % round up
k_spot = k; % which graph from the graph grid
k_figure = 0; % which number
while k_spot > k_rows * k_rows % If it does not fit, start all over.
    k_spot = k_spot - k_rows * k_rows;
    k_figure = k_figure + 10;
end

duration_s = (1/filter):(1/filter):(duration/filter); % zero does not exist in matlab, therefore it starts at the smallest point.
% 1/filter first element in the graph or matrix, 1/filter step size, and ...
% duration/filter last element in the graph/matrix
sweep_data = data(1:duration, 1, sweep); % sweep is an argument

figure(1 + k_figure);
subplot(k_rows, k_rows, k_spot);
plot(duration_s, sweep_data);
xlabel('Time (sec)');
ylabel('Voltage(mV)');
title(file);
set(figure(1 + k_figure), 'Visible', 'On');

thresh_AP = -30; % what threshold voltage needs to pass to be considered ...
as firing an AP

AP_times = zeros(10000, 1);
AP_times_shifted = zeros(10000, 1);
AP_max = -1000;
declining = 0;
AP_times_number = 0;
AP_sizes = zeros(10000, 1);
AP_min_list = zeros(100000,1);

for i = 1:duration
    if sweep_data(i) > thresh_AP || (declining == 0 && AP_max > thresh_AP)
        if declining == 0
            if sweep_data(i) > AP_max
                AP_max = sweep_data(i);
            else
                declining = 1;
                AP_times_number = AP_times_number + 1;
                AP_times(AP_times_number) = i - 1;
                AP_times_shifted(AP_times_number + 1) = i - 1;
                AP_sizes(AP_times_number) = AP_max;
            end
        end
    else
        if declining == 1 && sweep_data(i) > sweep_data(i-1) && ...
            AP_times_number > 0
            declining = 0;
            AP_min_list(AP_times_number) = sweep_data(i-1);
            AP_max = -10000;
        end
    end
end

AP_times=AP_times(2:AP_times_number);
AP_times_shifted=AP_times_shifted(2:AP_times_number);
AP_min_list=AP_min_list(1:AP_times_number);
frequency = AP_times_number / (duration / filter);

ISI = AP_times - AP_times_shifted;

ISI_values = ISI / filter;

AP_sizes = AP_sizes(1:AP_times_number);

AP_actual_sizes = AP_sizes - AP_min_list;

CV = std(ISI_values) / mean(ISI_values);

title_pos = strcat(ExcelCol(k), '1');

efreq_pos = strcat(ExcelCol(k), '2');

mean_pos = strcat(ExcelCol(k), '3');

CV_pos = strcat(ExcelCol(k), '4');

data_pos = strcat(ExcelCol(k), '6');

eexcel_name = sprintf('%s\Frequency_%s.xlsx', location, date);

xlswrite(excel_name, {filename}, 1, title_pos{1});

xlswrite(excel_name, frequency, 1, freq_pos{1});

xlswrite(excel_name, mean(ISI_values), 1, mean_pos{1});

xlswrite(excel_name, CV, 1, CV_pos{1});

xlswrite(excel_name, ISI_values, 1, data_pos{1});

figure(2);

for j = 1:length(ISI_values)

    line([ISI_values(j) ISI_values(j)], [k-1 k]);
end

xlabel('Time (sec)'); ylabel('Trial no.');

set(figure(2), 'Visible', 'On');

figure(3 + k_figure);

subplot(k_rows, k_rows, k_spot);

lnISI = log10(ISI_values);

hist(lnISI, 50);

xlabel('10^\');

ylabel('Number of Occurences');

title({filename});
buckets = 250;
lags = 25000;
bucketsize = lags / buckets;

if length(sweep_data) ≥ lags
    lags = length(sweep_data) - 1;
end

a = autocorr(sweep_data, lags);
b = zeros(buckets,1);

for i = 0:(buckets-1)
    b(i+1) = mean(a((i*bucketsize+1):((i+1)*bucketsize),1));
end

%figure(4 + k_figure);
%subplot(k_rows,k_rows,k_spot);
%bar(b);
%title({'file ' 'Autocorr'});
%set(figure(4 + k_figure), 'Visible', 'On');
end
A.3 Current step analysis with automatically determined action potential threshold

A.3.1 Current steps analysis script

```matlab
close all

show_figures = 0;
path = fileparts(mfilename('fullpath'));
delete(sprintf('%s/Output/Current_steps/*.xlsx', path));
addpath(sprintf('%s/Includes', path));

[filenames, path] = uigetfile({'*.abf', 'Select_file(s)', ...
    'MultiSelect', 'on'});

if ~iscell(filenames)
    filenames = {filenames}; %make it into one element array
end

number_of_files = length(filenames); %length is a function getting a ...

m = 1;

for i = 1:number_of_files
    fullname = strcat(path, filenames(i));
    data = abfload(fullname{1});
    name = filenames(i);

    if isempty(data)
        continue
    end

    AP_sizes_list, AP_actual_sizes_table,...
    AP_number]=CurrentStepsFunction(data, name{1}, 'Current_Steps', ...
    m, show_figures, path, filenames{1});
```

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A.3.2 Current steps function

```matlab
function [AP_sizes_list, AP_actual_sizes_table, ...
    potential_AP_number]=CurrentStepsFunction(data, filename, ...
    output_folder, m, show_figures, location, name)

if nargin < 5
    show_figures = 1;
end

k_rows=4;
duration_start = 115000;
duration_end = 215500;
duration = duration_start:duration_end;
duration_s=duration/100000;
sweeps=size(data,3);
step_number=(1:sweeps)';

ISI_average_list=zeros(sweeps,1);
AP_average_list=zeros(100,1);
AP_actual_sizes_averages_list=zeros(100,1);
current_injection = -50:50:700;

figure(1 + m * 10);
set(1 + m * 10, 'Name', filename);
hold on
set (figure(1+m*10), 'visible','off');
for j=1:size(data,3)
    subplot(k_rows,k_rows,j);
    plot(duration_s,data(duration,1,j));
    xlabel('Time (sec)');
end
```
ylabel('Voltage (mV)');
end

frequency_list=zeros(sweeps,1);
ISI_values_list=zeros(100,sweeps);
AP_sizes_list=zeros(100,sweeps);
AP_actual_sizes_table=zeros(100,sweeps);
AP_normalised=zeros(sweeps,1);
AP_actual_sizes_averages_table=zeros(100,sweeps);

for j=1:sweeps
    AP_times=zeros(100,1);
    ISI=zeros(100,1);
    potential_AP_times=zeros(500,1);
    potential_AP_number=0;
    AP_number=0;
    AP_sizes=zeros(100,1);
    sweep_data=data(1:size(data,1),1,j);
    fire = 0;
    
    if j==1
        baseline_mean=mean(data(1:115000));
        step_mean=mean(data(150000:210000));
        input_resistance=(baseline_mean-step_mean)/50*1000;
    end

    mavg = 0;
    mavgcount = 0;
    AP_min_last = -1000;
    AP_max_last = 1000;
    AP_last_real = 0;

    for i = duration
        mavg = mavg + sweep_data(i-1);
        mavgcount = mavgcount + 1;
        if mavgcount > 100

mavg = mavg - sweep_data(i-101);
mavgcount = mavgcount - 1;

end

if sweep_data(i) > mavg / mavgcount || i == duration_end ...

%increasing

if fire == 0
    fire = 1;
    if potential_AP_number > 0
        peak = i - mavgcount;
        for n = 1:(mavgcount - 1)
            if sweep_data(i - mavgcount + n) < sweep_data(peak)
                peak = i - mavgcount + n;
            end
        end
    end
end

AP_min = sweep_data(peak);
AP_size = AP_max - AP_min;
AP_min_last = AP_min;
AP_last_real = 0;

if AP_size > 10 && (AP_number == 0 || AP_size > 0.5 ...) 
* AP_sizes(AP_number) || AP_size > 15)
    AP_number = AP_number + 1;
    AP_last_real = 1;
    AP_sizes(AP_number) = AP_size;
    AP_times(AP_number) = ...
    potential_AP_times(potential_AP_number);
    if AP_number > 1
        ISI(AP_number - 1) = AP_times(AP_number) - ...
        AP_times(AP_number - 1);
    end
end
end

elseif sweep_data(i) < mavg / mavgcount
    if fire == 1

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peak = i − mavgcount;

for n = 1:(mavgcount − 1)
    if sweep_data(i − mavgcount + n) > sweep_data(peak)
        peak = i − mavgcount + n;
    end
end

if sweep_data(peak) − AP_min_last < 2
    fire = 0;
    AP_max = AP_max_last;
    if AP_last_real
        AP_number = AP_number − 1;
    end
    else
        potential_AP_number = potential_AP_number + 1;
        fire = 0;
        AP_max = sweep_data(peak);
        AP_max_last = AP_max;
        potential_AP_times(potential_AP_number) = peak;
    end
end

end

if AP_number < 1
    continue
end

if AP_number > 1
    ISI = ISI(1:(AP_number−1));
    mean_ISI = mean(ISI);
    for n = 1:(AP_number − 1)
        if ISI(n) > mean_ISI ⋆ 3 && AP_sizes(n)<30;
            AP_number = n;
            ISI = ISI(1:n−1);
            break
    end
end
end

end

ISI_number=AP_number-1;
ISI_values=ISI/100000;
ISI_values_list(1:length(ISI_values),j)=ISI_values;
ISI_average=(sum(ISI_values))/ISI_number;
ISI_average_list(j)=ISI_average;

AP_sizes=AP_sizes(1:AP_number);
AP_actual_sizes_average=mean(AP_sizes); %AP average size for a sweep
AP_actual_sizes_averages_list(j)=AP_actual_sizes_average;
AP_normalised(j)=mean(AP_sizes./AP_sizes(1));
frequency=AP_number;
frequency_list(j)=frequency;

AP_sizes_list(1:AP_number,j)=AP_sizes;
AP_actual_sizes_table(1:(AP_number),j)=AP_sizes;
AP_average=mean(AP_sizes);
AP_average_list(j)=AP_average;

if AP_number > 0
   AP_actual_sizes_averages_table(1:AP_number,...
       j)=AP_sizes./AP_sizes(1);
end

if show_figures
   if AP_times_number > 1
      figure(2 + m * 10);
      set(2 + m * 10, 'Name', filename);
      hold on
      for k=1:(length(ISI_values)-1);
         line([ISI_values(k) ISI_values(k)], [j-1 j]);
      end
      xlabel('ISI duration (sec)'); ylabel('#Sweep');
end

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figure (3 + m * 10);
set (3 + m * 10, 'Name', filename);
subplot (k_rows, k_rows, j);
plot (ISI_values);
xlabel ('#ISI in a sweep');
ylabel ('Duration (sec)');
end

figure (4 + m * 10);
set (4 + m * 10, 'Name', filename);
subplot (k_rows, k_rows, j);
if potential_AP_number > 0
  plot ((AP_sizes./AP_sizes(1)));
end
xlabel ('#AP in a sweep');
ylabel ('Normalized peaks');

figure (5 + m * 10);
set (5 + m * 10, 'Name', filename);
subplot (k_rows, k_rows, j);
plot (AP_actual_sizes);
xlabel ('#AP in a sweep');
ylabel ('Actual size (mV)');

figure (6 + m * 10);
set (6 + m * 10, 'Name', filename);
subplot (k_rows, k_rows, j);
if potential_AP_number > 0
  plot ((AP_actual_sizes./AP_actual_sizes(1)));
end
xlabel ('#AP in a sweep');
ylabel ('Normalised (actual)');
end
ISI_values_list_filtered = ISI_values_list;
ISI_values_list_filtered = nan;

AP_actual_sizes_averages_table_filtered = AP_actual_sizes_averages_table;
AP_actual_sizes_averages_table_filtered = nan;

AP_average_list = AP_average_list(1:(sweeps));
AP_actual_sizes_averages_list = AP_actual_sizes_averages_list(1:(sweeps));

if show_figures
    figure(7 + m * 10);
    set(7 + m * 10, 'Name', filename);
    scatter((current_injection), frequency_list./10);
    xlabel('Injected current (pA)');
    ylabel('Frequency (Hz)');

    figure(8 + m * 10);
    set(8 + m * 10, 'Name', filename);
    scatter(current_injection, ISI_average_list);
    xlabel('Injected current (pA)');
    ylabel('Mean ISI (sec)');

    figure(9 + m * 10);
    set(9 + m * 10, 'Name', filename);
    scatter(current_injection, AP_average_list);
    xlabel('Injected current (pA)');
    ylabel('AP peak (mV)');

    figure(10 + m * 10);
    set(10 + m * 10, 'Name', filename);
    scatter(current_injection, AP_actual_sizes_averages_list);
    xlabel('Injected current (pA)');
    ylabel('Actual action potential size (mV)');

warning('off', 'MATLAB:xlswrite:AddSheet');
excel_name = sprintf('%s\current_steps_%s.xlsx', location, date); %it...
    tells the full path of the file
xlswrite(excel_name, {filename}, m, 'A1');
xlswrite(excel_name, {'Step'}, m, 'B1');
xlswrite(excel_name, step_number, m, 'B2');
xlswrite(excel_name, {'Current'}, m, 'C1');
xlswrite(excel_name, current_injection, m, 'C2');
xlswrite(excel_name, step_number, m, 'B2');
xlswrite(excel_name, current_injection, m, 'C2');
xlswrite(excel_name, {APs}, m, 'D1');
xlswrite(excel_name, frequency_list, m, 'D2');
average_frequencies = 1 ./ ISI_average_list;
average_frequencies(~isfinite(average_frequencies)) = 0;
xlswrite(excel_name, {'AV frequency'}, m, 'E1');
xlswrite(excel_name, average_frequencies, m, 'E2');
xlswrite(excel_name, input_resistance, m, 'E19');
xlswrite(excel_name, {'AP actual size'}, m, 'F1');
xlswrite(excel_name, AP_actual_sizes_averages_list, m, 'F2');
xlswrite(excel_name, {AP normalised}, m, 'G1');
xlswrite(excel_name, AP_normalised, m, 'G2');
xlswrite(excel_name, {'Average ISI'}, m, 'H1');
xlswrite(excel_name, ISI_average_list, m, 'H2');
excel_name = sprintf('%s\ISI_values_%s.xlsx', location, date);
xlswrite(excel_name, {filename}, m, 'A1');
xlswrite(excel_name, {'ISI values'}, m, 'A3');
xlswrite(excel_name, transpose(step_number), m, 'B3');
xlswrite(excel_name, {'Step'}, m, 'B2');
xlswrite(excel_name, ISI_values_list_filtered, m, 'B4');
excel_name = sprintf('%s\AP_normalised_%s.xlsx', location, date);
xlswrite(excel_name, {filename}, m, 'A1');
xlswrite(excel_name, {'Normalised AP'}, m, 'A3');
xlswrite(excel_name, transpose(step_number), m, 'B3');
275 `xlsxwrite(excel_name, {'Step'}, m, 'B2');
276 `xlsxwrite(excel_name, AP_actual_sizes_averages_table_filtered, m, 'B4');
277
278 excel_name = sprintf("%s\AP_actual_sizes_%s.xlsx", location, date);
279 `xlsxwrite(excel_name, {filename}, m, 'A1');
280 `xlsxwrite(excel_name, {'Actual AP'}, m, 'A3');
281 `xlsxwrite(excel_name, transpose(step_number), m, 'B3');
282 `xlsxwrite(excel_name, {'Step'}, m, 'B2');
283 `xlsxwrite(excel_name, AP_actual_sizes_table_filtered, m, 'B4');
284
285 title_pos = strcat(ExcelCol(m+1), '1');
286 freq_pos = strcat(ExcelCol(m+1), '2');
287 input_pos = strcat(ExcelCol(m+1), '19');
288
289 excel_name = sprintf("%s\CS APs summary_%s.xlsx", location, date);
290 `xlsxwrite(excel_name, {'Current'}, 1, 'A1');
291 `xlsxwrite(excel_name, current_injection', 1, 'A2');
292 `xlsxwrite(excel_name, {filename}, 1, title_pos{1});
293 `xlsxwrite(excel_name, frequency_list, 1, freq_pos{1});
294
295 excel_name = sprintf("%s\CS AP normalised summary_%s.xlsx", location, date);
296 `xlsxwrite(excel_name, {'Current'}, 1, 'A1');
297 `xlsxwrite(excel_name, current_injection', 1, 'A2');
298 `xlsxwrite(excel_name, {filename}, 1, title_pos{1});
299 `xlsxwrite(excel_name, AP_normalised, 1, freq_pos{1});
300
301 excel_name = sprintf("%s\CS freq summary_%s.xlsx", location, date);
302 `xlsxwrite(excel_name, {'Current'}, 1, 'A1');
303 `xlsxwrite(excel_name, current_injection', 1, 'A2');
304 `xlsxwrite(excel_name, {filename}, 1, title_pos{1});
305 `xlsxwrite(excel_name, average_frequencies, 1, freq_pos{1});
306 `xlsxwrite(excel_name, {'Input resistance'}, 1, 'A19');
307 `xlsxwrite(excel_name, input_resistance, 1, input_pos{1});
308
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Appendix B

List of Publications

B.1 Journal Articles


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B.2 Abstracts

*International Ataxia Research Conference, 2015.*
Suminaite D, Perkins E, Clarkson Y and Jackson M. Elucidating the reversibility of ataxia as a basis for development of therapies.

*Physiological Society Meeting, 2015.*
Suminaite D, Perkins E, Lee C, Clarkson Y and Jackson M. Elucidating the Reversibility of Ataxia.

*10th Federation of Neurosciences Societies (FENS) Forum, 2016*
Suminaite D, Perkins E, Lee C, Clarkson Y and Jackson M. Elucidating the Reversibility of Ataxia.

B.3 Posters

Recessive Mutations in SPTBN2 Implicate β-III Spectrin in Both Cognitive and Motor Development

Stefano Lise1,2,*, Yvonne Clarkson2*, Emma Perkins3*, Alexandra Kwasniewska1,4, Elham Sadighi Akha1,2, Ricardo Parolin Schnekenberg1,5, Daumante Suminaite3, Jilly Hope5, Ian Baker6, Lorna Gregory1, Angie Green1, Chris Allan1, Sarah Lamb1, Sandeep Jayawant7, Gerardine Quaghebeur8, M. Zameel Cader9, Sarah Hughes10, Richard J. E. Armstrong4,10, Alexander Kanapin1, Andrew Rimmer1, Gerton Lunter1, Iain Mathieson1, Jean-Baptiste Cazier1, David Buck1, Jenny C. Taylor1,2, David Bentley11, Gilean McVean1, Peter Donnelly1, Samantha J. L. Knight1,2, Mandy Jackson3*, Siannis Ragoussi1, Andrea H. Németh1,2,4,12*.

1 Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom, 2 NIHR Biomedical Research Centre Oxford, Oxford, United Kingdom, 3 Centre for Integrative Physiology, Euan MacDonald Centre for Motor Neurone Disease Research, University of Edinburgh, Edinburgh, United Kingdom, 4 Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom, 5 School of Medicine, Universidade Positivo, Curitiba, Brazil, 6 Russell Cairns Unit, Oxford University Hospitals NHS Trust, Oxford, United Kingdom, 7 Department of Paediatrics, Oxford University Hospitals NHS Trust, Oxford, United Kingdom, 8 Department of Neuroradiology, Oxford University Hospitals NHS Trust, Oxford, United Kingdom, 9 Department of Anatomy, Physiology, and Genetics, University of Oxford, Oxford, United Kingdom, 10 Royal Berkshire Foundation Trust Hospital, Reading, United Kingdom, 11 Illumina Cambridge, Saffron Walden, United Kingdom, 12 Department of Clinical Genetics, Oxford University Hospitals NHS Trust, Oxford, United Kingdom.

Abstract

β-III spectrin is present in the brain and is known to be important in the function of the cerebellum. Heterozygous mutations in SPTBN2, the gene encoding β-III spectrin, cause Spinocerebellar Ataxia Type 5 (SCA5), an adult-onset, slowly progressive, autosomal-dominant pure cerebellar ataxia. SCAS is sometimes known as “Lincoln ataxia,” because the largest known family is descended from relatives of the United States President Abraham Lincoln. Using targeted capture and next-generation sequencing, we identified a homozygous stop codon in SPTBN2 in a consanguineous family in which childhood developmental ataxia co-segregates with cognitive impairment. The cognitive impairment could result from mutations in a second gene, but further analysis using whole-genome sequencing combined with SNP array analysis did not reveal evidence of other mutations. We also examined a mouse knockout of β-III spectrin in which ataxia and progressive degeneration of cerebellar Purkinje cells has been previously reported and found morphological abnormalities in neurons from prefrontal cortex and deficits in object recognition tasks, consistent with the human cognitive phenotype. These data provide the first evidence that β-III spectrin plays an important role in cortical brain development and cognition, in addition to its function in the cerebellum; and we conclude that cognitive impairment is an integral part of this novel recessive ataxic syndrome, Spectrin-associated Autosomal Recessive Cerebellar Ataxia type 1 (SPARCA1). In addition, the identification of SPARCA1 and normal heterozygous carriers of the stop codon in SPTBN2 provides insights into the mechanisms of molecular dominance in SCA5 and demonstrates that the cell-specific repertoire of spectrin subunits underlies a novel group of disorders, the neuronal spectrinopathies, which includes SCA5, SPARCA1, and a form of West syndrome.


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* E-mail: andrea.nemeth@eye.ox.ac.uk (AH Németh); mandy.jackson@staffmail.ed.ac.uk (M Jackson)

† These authors contributed equally to this work.

Introduction

Spectrins are a diverse family of membrane scaffold proteins. They were originally found in erythrocytes where mutations result in various haemolytic anemias [1,2]. Spectrins have been identified in the brain [3] but until recently little was known of the effects in humans of brain spectrin mutations. In 2006, heterozygous mutations of the brain spectrin gene SPTBN2, encoding β-III spectrin, were found to cause Spinocerebellar Ataxia Type 5 (SCA5) [4]. SCA5 is an autosomal dominant, slowly progressive, adult onset, pure cerebellar ataxia, which was first identified in a large family who are the descendents of relatives...
Recessive Mutations in SPTBN2

Author Summary

β-III spectrin is present in the brain and is known to be important in the function of the cerebellum. Mutations in β-III spectrin cause spinocerebellar ataxia type 5 (SCA5), sometimes called Lincoln ataxia because it was first described in the relatives of United States President Abraham Lincoln. This is generally an adult-onset progressive cerebellar disorder. Recessive mutations have not previously been described in any of the brain spectrins. We identified a homozygous mutation in SPTBN2, which causes a more severe disorder than SCA5, with a developmental cerebellar ataxia, which is present from childhood; in addition there is marked cognitive impairment. We call this novel condition SPARCA1 (Spectrin-associated Autosomal Recessive Cerebellar Ataxia type 1). This condition could be caused by two separate gene mutations; but we show, using a combination of genome-wide mapping, whole-genome sequencing, and detailed behavioural and neuropathological analysis of a β-III spectrin mouse knockout, that both the ataxia and cognitive impairment are caused by the recessive mutations in β-III spectrin. SPARCA1 is one of a family of neuronal spectrinopathies and illustrates the importance of spectrins in brain development and function.

of the US President Abraham Lincoln; SCA5 is therefore sometimes referred to as “Lincoln ataxia” [5,6,7]. Two other SCA5 families have been described in the literature, one from France and one from Germany [8,9].

β-III spectrin is a 2,390 amino acid protein comprising an N terminal domain containing the actin/ARP1 binding site, 17 spectrin repeats, (the latter containing regions which bind the glutamate transporter EAAT4 [10], and ankyrin [11]), and a C terminal domain of uncertain function. β-III spectrin forms antiparallel tetrameric heterodimers with α-II spectrin, encoded by SPTAN1. The tetrameric self-association probably requires the presence of the C terminal β spectrin repeats, B16 and B17, and the N terminal α spectrin repeats, A0 and A1, with absence of these regions highly likely to impair the formation of a functional tetramer [12]. Three heterozygous dominant mutations in SPTBN2 have been reported to cause SCA5: in the US (Lincoln) family a 13 amino acid in-frame deletion (E532_M544del) in the third spectrin repeat, in the French family a 13 amino acid in-frame deletion-insertion (L629_R634delinsW), also in the third spectrin repeat, and in the German family a missense mutation (L253P), in the N terminal domain. The mechanism of action of these mutations is not immediately obvious and could be explained by haploinsufficiency, in which the mutant allele is inactive and the normal stoichiometry for tetramer formation is lost, a dominant negative effect which suppresses wild type (wt) function, or a gain of function effect. Several lines of evidence have suggested that a dominant negative effect in SCA5 is most likely. Using targeted gene disruption of mouse β-III spectrin, Perkins et al, reported that homozygous knockout mice (β-III spectrin −/−) had cerebellar ataxia, a progressive loss of cerebellar Purkinje cells and an associated decrease in the Purkinje cell specific glutamate transporter EAAT4 [13]. The β-III spectrin −/− mutant mice lack all full-length β-III spectrin but do express, at a low level, a form of β-III spectrin (~250 KDa) that lacks most of the actin-binding domain encoded by exons 2–6. The heterozygous mice (β-III spectrin +/−) were reported to be normal. Further work has shown that the L253P (German) missense mutation has a dominant negative effect on wild type function by preventing protein trafficking from the Golgi apparatus [14]. There is evidence also that de novo in-frame mutations in SPTAN1 encoding α-II spectrin have dominant negative effects, causing a form of West Syndrome (infantile epilepsy with developmental delay) [15]. However, although experimental data has strongly suggested that small in-frame mutations or missense mutations in α-II or β-III spectrins have a dominant negative effect, no recessive mutations in spectrins have been found, and such data would lend further strong support for this hypothesis.

Here we report the first description of recessive mutations in SPTBN2 in which there is a severe developmental childhood ataxia but also significant cognitive impairment. The homozygous stop codon c.1881C>A (p.C627X), was identified in three affected individuals from a consanguineous family using targeted capture and next generation sequencing and both the ataxia and cognitive impairment co-segregate with the mutation. However, since more than one mutation can co-segregate, particularly in consanguineous families, we considered whether a second recessive mutation, either homozygous or compound heterozygous, could account for the cognitive impairment. We investigated this using a combination of SNP array analysis and whole genome sequencing, but found no evidence of a second mutation.

We also investigated β-III spectrin −/− knockout mice [13] for supportive evidence that the cognitive impairment in the human subjects is caused by loss of β-III spectrin. We examined the mouse model for morphological abnormalities of neurons in brain regions (other than cerebellum), which are thought to be involved in memory function including prefrontal cortical (PFC) layers, the caudate putamen/striatum and hippocampus (HPC). Finally we tested the mice using object recognition tasks, which have been shown to correlate with function of the PFC and HPC [16,17]. The morphological and behavioural abnormalities found in the knockout mice provide further evidence that the cognitive impairment in our human subjects is an integral part of this novel recessive disorder which we have called SPARCA1 (“Spectrin-associated Autosomal Recessive Cerebellar Ataxia type 1”). We suggest that this represents one of a novel group of disorders, the neuronal spectrinopathies, which demonstrate that the cell-specific functional repertoire of spectrin subunits are involved in brain development including the cortex, in addition to cerebellar development and function.

Results

Clinical phenotype and genetic analysis

The three affected individuals are from a UK family of Pakistani origin with complex consanguinity (see Figure 1A), but no other family history of neurological disorders. The clinical phenotype in the 3 individuals is identical (Table 1). V1 was referred at the age of 13 months with motor delay; she was extremely floppy and was unable to crawl. She sat at 10 months, crawled at 18 months and was pulling to stand at 20 months. She walked with a walker by the age of 5 and started to walk with support at age 7. She was noted to have language delay and at age 5 was just starting to join words together. Global developmental delay was subsequently noted, she was educated at a special school and now attends a college for adults with special educational needs. On examination there are abnormal eye movements with a convergent squint, hypometric saccades, jerky pursuit movements, and an incomplete range of movement particularly in the horizontal plane. There is obvious dysmetria and dysdiadochokinesia of the limbs and gait ataxia with inability to tandem walk without falling. Limb tone is normal, reflexes are normal and plantars flexor and there is no evidence of any sensory abnormality. Romberg's sign is normal. Neuropsy-
chological assessment reveals significant global cognitive impairment with all IQ scales falling at the second percentile or below, and with Full Scale IQ scores falling in the learning disabled range (Table 1). A brain CT scan at age 2 did not show any abnormality, but a recent MRI brain reveals significant cerebellar atrophy (Figure 2A). V2 is the younger sibling of V1. She was noted to have developmental delay in early childhood and also did not start to walk until age 7. On examination, she has an identical clinical phenotype to that of her sister except for occasional beats of nystagmus on eye examination. She attends a school for children with learning disabilities and a recent assessment (at age 16) shows functioning in English and Mathematics at the level of an average 5–7 year old in the UK requiring special educational support. Formal cognitive assessment also showed very similar impairments to V1 with scores on all IQ scales falling at the second percentile or below, and with Full Scale IQ scores falling in the learning disabled range (Table 1). The difference between Verbal and Performance IQ for each individual was not statistically significant (p = 0.15). MRI imaging in V2 at age 6 revealed cerebellar atrophy and this was found to have progressed over time (Figure 2Bi and Bii). V3 is the first cousin of V1 and V2. He was noted to have poor head control and balance in early childhood. Clinical examination is identical to his cousins and also shows an identical developmental profile in that he has just started to walk with assistance at the age of 7. He also has an identical eye movement disorder, a convergent squint, dysmetria and dysdiadochokinesia. He is hypotonic with normal reflexes downgoing plantars and no evidence of a sensory neuropathy. He attends a mainstream school but requires full time one to one support. Cognitive assessment of V3 also showed significant global cognitive impairment (Table 1). The slightly higher IQ scores in V3 results from a floor effect in the normative data rather than a significant difference in cognitive ability from his older cousins. In this age cohort the lowest attainable scores are VIQ = 62, PIQ = 73 and FSIQ = 63 and therefore V3 falls in the same learning disabled range as his cousins. Brain imaging of V3 showed a normal cerebellum at age 5, but mild hypoplasia of the posterior corpus callosum (Figure 2C). The normal appearance of the cerebellum in V3 at an early age is not unexpected as both his cousins imaging shows progression with time. Neurological examination of both sets of parents was entirely normal, with no evidence of ataxia. The father of V1 and V2 works as a bus driver, having left school at age 16 with 5 GCSEs (General Certificates of Secondary Education) and the father of V3 works in a warehouse and has a similar educational background. Formal psychometric testing in the father of V1 and V2 showed IQ indices falling in the low average range consistent with his educational attainment. The father of V3 was not available for testing but has very similar attainment levels to his brother. Formal assessment of the mothers could not be performed since neither speak English, but interview of the family did not reveal any evidence of learning disability. There is no history of the siblings or grandparents of the affected individuals having any cognitive or neurological abnormalities.

We initially performed targeted capture of >100 known ataxia genes (including SPTBN2) in a group of children with unexplained ataxia including patient V3, followed by next generation sequencing. In V3 we identified only one mutation, a homozygous stop codon p. C627X (c.1881C > A), located in the third spectrin repeat in SPTBN2 and used Sanger sequencing to confirm that all three affected patients in the family had the same mutation whereas the neurologically normal parents of V3, were shown to be heterozygous for the mutation (Figure 1B). Since mutations in β-III spectrin are associated with cerebellar degeneration in SCA5, the newly identified mutation was considered likely to explain the

![Figure 1. Genetic analysis of family with ataxia and cognitive impairment. A. Pedigree of family. B. Sanger sequencing of the mutation c. 1881C>A; p.C627X in normal, parents of V3 (IV3 and IV4) and affecteds, V1, V2, V3.](https://www.plosgenetics.org/doi/10.1371/journal.pgen.1003074.g001)
### Table 1. Clinical and neuropsychological assessments of family.

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<th>V2 (aged 16)</th>
<th>V3 (aged 7)</th>
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<th>IV2 (Mother of V1 and V2)</th>
<th>IV3 (Father of V3)</th>
<th>IV4 (Mother of V3)</th>
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<td>56 (0.2)</td>
<td>73 (4)</td>
<td>84 (14)</td>
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<td>53 (0.1)</td>
<td>67 (1)</td>
<td>80 (9)</td>
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ataxia, although of a developmental type with a much earlier onset. However, since more than one mutation can co-segregate, particularly in consanguineous families, we went on to consider the contribution of the mutation in SPTBN2 to the observed cognitive impairment. We therefore used SNP array analysis and whole genome sequencing to search for any evidence of a second mutation.

**SNP array genotyping**

To investigate whether a second homozygous mutation segregated with the cognitive impairment, all 3 affected individuals (V1, V2 and V3) and the unaffected parents of V3 (IV3 and IV4) were genotyped to identify regions of homozygosity (ROH) shared by V1, V2 and V3 and not present in either IV3 or IV4. This analysis identified 20 shared homozygous segments on autosomes totalling 17.1 Mb (Table 2). SPTBN2, on chromosome 11, was located in the largest ROH shared by V1, V2 and V3 and not present in either IV3 or IV4 (Figure 3).

**Whole-genome sequencing**

Whole genome sequencing of patient V2 was performed on the Illumina HiSeq2000 as 100 bp paired end reads, using v3 clustering and sequencing chemistry. After duplicate reads removal, the mean coverage across the genome was 25.6× with 90.4% of bases covered at 15× or more. The mean coverage over the 17.1 Mb ROH identified by SNP analysis was 25.9× with 93.4% of bases covered at 15× or more. Variant calling was performed as detailed in the Materials and Methods.

We firstly based our data analysis on an autosomal recessive disease model, caused by one or more rare homozygous mutations and focused on homozygous variants occurring in the shared ROH identified by SNP array analysis, filtering them out if they were:

- present in 1000 Genomes with an allele frequency >1% [http://www.1000genomes.org/]
- in a region of segmental duplication

**Figure 2. Neuroimaging of patients.** A. Sagittal T1w MRI in subject V1 age 21 demonstrating clear cerebellar atrophy. B. Sagittal T1w MRI in subject V2 at age 6. Sagittal T1w MRI in subject V2 age 16 shows clear atrophy of the cerebellum. C. Sagittal T1w MRI in subject V3 showing hypoplasia of posterior corpus callosum (white arrow).

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observed as homozygous in other WGS500 samples within our Institute (see Materials and Methods). These filtering steps identified 68 candidate variants, subdivided into functional classes (Table 3). Only 2 exonic variants were found: a synonymous variant, L551L on chr2 which is not predicted to be pathogenic and is not located near a splice site, and the stop codon C627X in SPTBN2 on chr11 (Table 2 and Table 3).

Of the remaining variants, 21 were intergenic and also considered unlikely to be disease related, and 4 variants were in untranslated regions (5’ UTR) or in non-coding RNAs and all were in positions which scored poorly with PhyloP and GERP. In addition, none of the associated genes (UBIAD1, LINC01309, LOC100130987) appear to be relevant for this disorder. The other 41 were in intronic and upstream regions but based on evolutionary conservation and available information in databases (eg HGMD [18]) we found no evidence of potential involvement in the disease. The only likely pathogenic variant is the stop codon in SPTBN2.

We also considered a model of recessive inheritance with compound heterozygous mutations segregating with the ataxia and/or cognitive impairment. Our criteria were that all 3 affecteds must have two different variants in the same gene and where this occurred the variants should be in cis (ie each parent is a carrier). We identified all potential compound heterozygous coding variants present in the WGS data for individual V2. In total there were variants fulfilling our criteria at 13 different loci but in only 1 case were both variants present in all 3 affecteds and further analysis revealed that in this instance both variants were also in the father of V3 (ie were in cis). Furthermore, none of the variants identified are known to be associated with ataxia or cognitive impairment and the majority of genes had data suggesting an alternative function (such as taste or fertility), nor were there any likely candidates based on pathogenicity bioinformatic prediction programs (Table S2).

Abnormal dendritic morphology of prefrontal cortical neurons in β-III spectrin −/− mice

The phenotype of our patients suggested that β-III spectrin is involved in cognitive development, in addition to being essential for motor functions. We therefore utilised β-III spectrin knockout mice which have progressive cerebellar degeneration and lack any full length β-III spectrin [13], to further investigate the role of β-III spectrin in other brain regions. Our previous work revealed that β-III spectrin is required for the correct dendritic development of Purkinje cells [19,20] and therefore we initially examined dendritic organisation in other brain regions by immunostaining sagittal sections from the brains of 6-week-old wild-type and β-III spectrin knockout animals when the cortex and striatum were immunostained for tau or myelin basic protein (MBP) indicating that there was no change to axonal structure (Figure S1).

The PFC in humans is believed to be important for complex cognitive tasks, and given there is evidence of a close association between this area and the neocerebellum, as well as high
expression levels of β-III spectrin in mouse [10] we further investigated the prefrontal cortical region in β-III spectrin knockout animals. There was no difference in the thickness of individual prefrontal cortical layers (data not shown) but the morphology of individual pyramidal neurons in β-III spectrin knockout animals was found to be altered. Morphometric analysis of dye-injected pyramidal neurons from layer 2/3 showed basal dendrites in 8-week-old β-III spectrin knockout mice were significantly thinner distally compared to wild type cells (Figure 4B–4D). Moreover, the basal dendrites of knockout mice tapered more rapidly than those of wild types, being significantly reduced in thickness between 20 and 30 μm from the soma, whereas wild type dendrites showed no significant narrowing until 90 μm from the soma. However, no difference in spine density was observed between genotypes in either dye injected (Figure 4D: +/-, 2.8±0.6, n = 8; −/−, 3.2±0.2 spine/μm², n = 7; p = 0.56) or Golgi-impregnated (Figure 4E: +/-, 12.4±1.7, n = 4; −/−, 13.7±1.3 spine/10 μm, n = 6; p = 0.56) pyramidal neurons. Only small sections of apical dendrites could be reconstructed from the serial stacks of dye-injected cells. Nevertheless, quantification of the short regions imaged, when normalized to length analysed, indicated reduced apical dendritic volumes, and hence thinner apical dendrites in β-III spectrin knockout animals (+/-, 4.3±0.47; −/−, 2.5±0.36 μm³/μm, n = 6 for each genotype; p = 0.011).

Corpus callosum appears normal in β-III spectrin −/− mice

Since patient V3 shows mild hypoplasia of the posterior corpus callosum we examined this brain structure in 8-week old β-III spectrin knockout animals to determine if the morphological defect in the human subject could be a consequence of β-III spectrin loss or is unlinked to the homozygous stop codon c.1881C>A (p.C627X) mutation in SPTBN2. No signs of posterior hypoplasia were observed in sagittal sections stained either with cresyl violet...
Figure 4. Abnormal dendritic morphology in β-III spectrin −/− mouse compared to wild type. A. Sagittal sections immunostained for MAP2 show irregular reactivity throughout prefrontal cortical layers and caudate putamen/striatum of 6-week-old β-III spectrin knockout (−/−) mice compared to wild type (+/+); but normal staining within hippocampus (N = 3 each genotype; Bar, 20 μm). B. Top, Representative images of pyramidal neurons in layer 2/3 prefrontal cortex from 8-week-old WT and β-III spectrin knockout mice filled with Alexa 568 (Bar, 20 μm). Bottom, Neuronal 3-D reconstruction overlay used using NeuronStudio software. C. Quantification of basal dendrite morphological parameters measured from reconstructed images shows greater distal thinning of dendrites in cells from β-III spectrin knockout mice (open circles; N = 9) compared with WT cells (filled squares; N = 8). All data are mean ± SEM (* denotes significance between groups and # significance within a group.) D. High magnification image of single axial dendrite showing distal thinning in β-III spectrin knockout compared to WT but presence of normal spines (Bar, 5 μm). E. High magnification image of Golgi impregnated pyramidal neuron from prefrontal cortex of WT and β-III spectrin knockout mice (Bar, 10 μm).

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β-III spectrin −/− mice are deficient in behaviour tasks

Four object recognition memory tasks (two- and four- novel object preference, object-in-place and object location; Figure 6A–6D) were carried out to assess whether β-III spectrin knockout animals displayed any cognitive deficits. No impairment in the two novel object recognition task (“object identity”) was observed in β-III spectrin knockout animals compared with wild type animals (Figure 6A); however knockout animals performed worse in the four novel object recognition task (Figure 6B). Knockout animals were also worse at discriminating between rearranged and non-rearranged objects in the object-in-place task compared with litter mate controls, shown by their failure to spend more time exploring the two objects in different locations compared with the two objects that had not moved (“object displacement”) (Figure 6C). However, there was no significant difference in performance for the object location task (Figure 6D). The poorer performance in the four-novel object recognition task for knockout animals was not a consequence of less exploration in the 5 minute sample phase as in fact they explored more than wild type animals (+/+; 64.9±6.7; −/−; 48.7±4.8 sec; p = 0.018). Similarly for the object-in-place task although there was no significant difference between genotypes there was a trend for greater exploration in knockout animals (+/+; 42±3.6; −/−; 62.2±8.7 sec; p = 0.054).

Discussion

The integrated evidence from clinical, genetic and neuropsychological analysis in humans and behavioural and morphological analysis in a mouse model demonstrate that we have identified a novel recessive disorder, SPARCA1, associated with mutations in β-III spectrin. The 3 human subjects with a premature stop codon...
and the mouse knockout all have very early onset cerebellar ataxia, indicating a developmental role for \( \beta\)-III spectrin. The human and mouse knockout phenotype also show that \( \beta\)-III spectrin is involved in cognitive development and function. The human subjects have global cognitive impairment in the mild/moderate range. The specific brain structures and connections associated with this impairment are not yet known and further detailed neuropsychological testing will be required. However, we have shown that in the mouse knockout there are morphological abnormalities especially thinning of dendrites in PFC neurons, similar to that previously reported for Purkinje neurons [19], but with no obvious changes in various regions of HPC (CA1, CA3 and dentate gyrus), and the behavioural tests in the mouse are consistent with this. Based on published lesion studies, deficits in the object-in-place task but not the object location task would indicate defects in the PFC not HPC, since PFC is believed to mediate memory for object location (Object displacement), whereas HPC integrates information as to object identity and the temporal order of object presentation with HPC lesioned animals being impaired on object location task [16,17,21]. However, further to the above discussion, there is also increasing recognition that the cerebellum itself has a direct role in cognition [22] and it is possible that some of the phenotype results directly from cerebellar abnormalities. Further investigation should also allow a detailed analysis of which specific brain regions mediate mild/moderate cognitive impairment in humans.

The data demonstrate that our \( \beta\)-III spectrin knockout mouse [13] is an excellent model for the novel recessive disorder we have identified and will allow further molecular analysis of \( \beta\)-III spectrin, in addition to the morphological and behavioural analysis. \( \beta\)-III spectrin is known to be expressed widely throughout the brain, kidney, liver and testes and to be associated with the Golgi and other cytoplasmic vesicles [25], but the mechanisms by which mutations lead to impaired brain development are unknown. The premature stop codon C627X identified in our family is predicted to result in truncation of \( \beta\)-III spectrin near the end of the 3rd spectrin repeat (Figure 7). This truncated protein would be unable to form tetramers with \( \alpha\)-II spectrin, nor be able to bind to EAAT4 or ankyrin, but it is possible that there is nonsense mediated decay and loss of the entire protein. Since \( SPTBN2 \) is expressed at only very low levels in peripheral blood, further in vitro expression studies will be required to determine this. However, it is most likely that \( \beta\)-III spectrin is absent in the brain of the human subjects and this has resulted in neuronal dysfunction in widespread brain regions, notably cerebellum and prefrontal cortex. Future studies will investigate other brain regions such as striatum and perirhinal cortex as well.

Our findings also provide insights into the mechanism of molecular dominance in SCA5: the heterozygous carrier parents of the C627X stop codon in the SPARCA1 family are neurologically normal despite carrying a stop codon which in the homozygous state is a recessive loss of function mutation. Therefore haploinsufficiency is highly unlikely to be the mechanism underlying SCA5 and this lends considerable weight to the body of experimental evidence suggesting that SCA5 results from a dominant negative effect, possibly by interfering with normal binding to ARP1 [13,14,24].

One difference between the human and mouse model is that the mouse shows progressive motor deficits in addition to progressive Purkinje cell loss whereas there is no evidence of clinical progression in the patients at the moment despite one of our subjects having progressive cerebellar atrophy on imaging. This lack of clinical progression and discordance between the clinical and imaging findings could suggest that there is significant plasticity within the human cerebellum, although we cannot exclude the possibility that slow clinical progression will occur with time.

The phenotypic spectrum of neuronal spinocerebellar ataxias now appears to be very wide. In SCA5, the ataxia is generally a pure adult-onset ataxia whereas recessive mutations in \( SPTBN2 \) cause SPARCA1, a more severe childhood ataxia with cognitive impairment. In West Syndrome, associated with \( SPTAN1 \) mutations, the patients have epilepsy, profound developmental delay and in addition have shortening of the corpus callosum and cerebellar vermis atrophy. Only one of our patients, V3, had shortening of the corpus callosum and it is tempting to speculate that this additional feature may be part of the SPARCA1 phenotype, although there are no signs of hypoplasia in the \( \beta\)-III spectrin knockout mice. It also may be that this feature is caused by another gene mutation or a genetic modifier and to clarify this additional cases will need to be identified. Overall, our data suggest that region specific expression of spectrin subunits is important in prenatal brain development and further work is required to define their temporal and spatial contribution.

Our data also suggest the possible and testable hypothesis that the phenotype in neuronal spinocerebellar ataxias relates in part to the total amount of functional spectrin tetramers: in SCA5, all \( \alpha\)-II/\( \beta\)-II tetramers are normal and functional but \( \alpha\)-II/\( \beta\)-III tetramers

Figure 5. Absence of hypoplasia of posterior corpus callosum in \( \beta\)-III spectrin knockout mice. Sagittal sections from 8-week old WT (+/+) and knockout (−/−) animals stained with cresyl violet (A, Bar 200 μm) and anti-tau antibody (B, Bar 500 μm) with arrow pointing to posterior corpus callosum. C. Coronal sections immunostained for MBP (Bar 200 μm).

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will contain mutant β-III spectrin which likely have a dominant negative action and may not be fully functional; in SPARCA1, a recessive disorder, there is complete loss of the tetramerisation site of β-III spectrin so there will be normal α-II/β-III tetramers but no functional α-II/β-III tetramers, whereas the heterozygotes who are effectively “haploinsufficient” have enough α-II/β-III tetramer

Figure 6. β-III spectrin knockout mice display deficits in some object recognition tasks. Diagram of task and performance of WT (+/+ ) and β-III spectrin knockout mice (−/− ) in the four object recognition tasks. Two-novel object recognition (A), four-novel object recognition (B), object-in-place (C) and object location task (D). All data are mean ± SEM (N = 6–9; * P < 0.05). doi:10.1371/journal.pgen.1003074.g006

Figure 7. Diagram of β-III spectrin/α-II spectrin tetramer. This is composed of 2 β-III spectrin and 2 α-II spectrin molecules and the location of the homozygous stop codon C627X in SPTBN2 causing SPARCA1 relative to dominant mutations in SPTBN2 and SPTAN1. Mutations are only shown in one of the two molecules. Loss or truncation of β-III is likely to prevent formation of normal tetramers. The glutamate transporter, EAAT4, binds near the C terminus of β-III.

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Recessive Mutations in SPTBN2

to be clinically normal; in West Syndrome, caused by in-frame dominant SPTAN1 mutations [15], the majority of both α-II/β-II and α-II/β-III tetramers are abnormal resulting in the most severe of the disorders to be described so far (Figure S2). This model would suggest that homozgyous loss of function α-II spectrin mutations might be more severe or lethal and a very recent report of an α-II knockout mouse supports this and it will be important to identify the equivalent human disorder [25]. There may be other disorders associated with human disease; dominant negative or recessive mutations in β-II and proteins interacting with brain spectrins may also have similar phenotypes. For example, a mouse knockout model of Ankyrin G, was reported to cause Purkinje cell degeneration [26] but a human phenotype has not yet been found. In addition, seizures are described in SPTAN1 mutations [15] and another β-III spectrin knockout [24] and it will be important to search for spectrin mutations in epilepsy patients.

In conclusion, the identification of recessive mutations in β-III spectrin provides evidence that the cell-specific repertoire of spectrin subunits underlies a novel group of disorders, the neuronal spectrinopathies, including SCAS, a dominant form of West Syndrome and SPARCA1. It is likely that other human disorders are caused by mutations in neuronal spectrins and searches for these are in progress. We also demonstrate the power of analysing complex phenotypes in consanguineous families by using whole genome sequencing, which was critical in establishing that both the ataxia and the cognitive impairment were caused by the same mutation and illustrate how the use of genome sequencing, even in single human families, can help provide mechanistic insights into disease.

Materials and Methods

Ethics on study participants and animal analysis

Our institutional ethics committee approved the study on human participants and specific consent was obtained to include whole genome analysis. All procedures involving analysis of mutant mice were carried out according to the United Kingdom Animals (Scientific Procedures) Act (1986) and other Home Office regulations under specific pathogen-free conditions.

Targeted resequencing and analysis

The exonic sequences of 129 genes known or suspected to be associated with ataxia were selected for targeted capture (Table S1) and 120-mer baits with 2X tiling designed using the Agilent eArray design tool. The total size of the targeted region amounted to 605.8 kb. Multiplex sequencing was performed on the Illumina GAII with 51 bp paired-end reads. A total of 5,046,154 reads were generated for patient V3 and aligned to the human reference genome (GRCh37/hg19) with the Illumina Inc. protocol. The data were transformed and quantified using the Illumina Inc. protocol and the dbSNP database (Build 135 [31]). We filtered out 18,000 markers which could not be mapped unambiguously to build 37 of the human genome. We further excluded SNPs with missing calls in one or more samples, thus reducing the number of markers to 271,208.

PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/ [32]) was used to identify regions of homozygosity (ROH) shared by V1, V2 and V3 and not present in either IV3 or IV4. For V1, V2 and V3, we applied relaxed parameters in order to include all potential ROH, resulting in potential false positives but minimizing false negatives. We defined a homozygous region as a run of (at least) 50 homozygous SNPs spanning more than 500 kb, allowing for some heterozygous calls within it. Shared ROH were identified from overlapping and allele matching segments. Further details of the algorithm are provided on the PLINK website. We used the options: --homozyg--homozyg-group--homozyg-window-snp 500--homozyg-window-sup 50--homozyg-kb 500. All other parameters were left at default values. ROH were then identified in IV3 and IV4. In this case very stringent criteria were applied to confidently include only true ROH and avoid false positives. We defined a homozygous region as an uninterrupted run of (at least) 500 homozygous SNPs spanning more than 5 Mb. In IV3 we identified 8 ROH on autosomes totalling 78 Mb (the largest ROH was 18.4 Mb); in IV4 we identified 2 large ROH on chromosome 11 present also in V1, V2 and V3 (Table 2 and Figure 3). These regions were excluded in the search for pathogenic variants as both IV3 and IV4 are unaffected. As a result, the search was restricted to 20 regions totalling 17.1 Mb, among which the ROH harbouring SPTBN2 was the largest.

Whole-genome sequencing

Data generation. Whole-genome sequencing of patient V2 was carried out as part of the WGS500 project, a collaboration between the University of Oxford and Illumina to sequence 3,000 whole genomes of clinical relevance. (http://investor.illumina.com/phoenix.zhtml?c=121127&p=irol-newsArticle&IID=1392299).

At time of writing 213 genomes have been completed and have been grouped and organised in the WGS500 Data Freeze 3 (February 2012).

Library preparation and sequencing. Samples were quantified using the High Sensitivity Qubit system (Invitrogen) and on a 3730xl DNA Sequencing Analyzer (Life Technologies). The sequence traces were aligned to the gene-specific reference sequence (NCBI build 37) with Sequencer 4.10.1 (Gene Codes).

SNP array genotyping and homozygosity mapping

Genotyping was performed using the Illumina HumanCytoSNP-12v1 BeadChip, containing nearly 300,000 genetic markers. Hybridization to the chip was performed according to manufacturer’s protocols found on registration at http://www.illumina.com/support/arrays/array_kits/humancyto-snp-12_v1/v2-1_dna_analysis_kit/documentation.illum. In brief, patient DNA was denatured, amplified and enzymatically fragmented and then hybridized onto CytoSNP-12 BeadChips by rockin in an Illumina hybridization oven at 48°C for 16-24 hrs. The BeadChips were washed according to the Illumina Inc. protocol and the hybridized DNA detected by primer extension with labelled nucleotides followed by detection using fluorescent antibodies. The data were processed using Illumina’s GenomeStudio V2009.2.

As SNP coordinates in the chip were reported with respect to human genome build 36, we downloaded the corresponding coordinates for build 37 from the website http://www.well.ox.ac.uk/~wrayner/strand/, cross-checking them using the USCS Genome Browser liftOver utility (http://genome.ucsc.edu/cgi-bin/hg/liftOver) and the dbSNP database (Build 135 [31]). We filtered out 18,000 markers which could not be mapped unambiguously to build 37 of the human genome. We further excluded SNPs with missing calls in one or more samples, thus reducing the number of markers to 271,208.

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Confirmation of variants using Sanger sequencing

Results were confirmed using Sanger Dideoxy Sequencing with the following primers across exon 14 of SPTBN2: Forward: CTACAACCCTGCCTGAGCACCT; Reverse: AGGGAG-GAAAGTCCAGAGAGA. Genomic DNA was amplified with Taq Polymerase (Roche) and PCR products were used as templates for sequencing with BigDye Terminator reagents (Life Technologies) and washed according to the Illumina Inc. protocol. The data were transformed and quantified using the Illumina Inc. protocol and the dbSNP database (Build 135 [31]). We filtered out 18,000 markers which could not be mapped unambiguously to build 37 of the human genome. We further excluded SNPs with missing calls in one or more samples, thus reducing the number of markers to 271,208.

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Data generation. Whole-genome sequencing of patient V2 was carried out as part of the WGS500 project, a collaboration between the University of Oxford and Illumina to sequence 300 whole genomes of clinical relevance. (http://investor.illumina.com/phoenix.zhtml?c=121127&p=irol-newsArticle&IID=1392299). At time of writing 213 genomes have been completed and have been grouped and organised in the WGS500 Data Freeze 3 (February 2012).

Library preparation and sequencing. Samples were quantified using the High Sensitivity Qubit system (Invitrogen) and
sample integrity was assessed using 1% E-Gel EX (Invitrogen). 2 ug of DNA were fragmented using the Covaris S2 system. Libraries were constructed using the NEBNext DNA Sample Prep Master Mix Set 1 Kit (NEB) with minor modifications. Ligation of adapters was performed using 6 ul of Illumina Adapters (Multiplexing Sample Preparation Oligoconecoict Kit). Ligated libraries were size selected using 2% E-Gel EX (Invitrogen) and the distribution of fragments in the purified fraction was determined using Tapestation 1DK system (Agilent/Lab901). Each library was PCR enriched with 25 uM each of the following custom primers: Multiplex PCR primer 1.0: 5'-3' Index primer: 5'-CAAGCGAGAAGGCGATACGATGAT|INDEXEX|CAGT-GACTGGAGTTCAGACGTGCTCTTGCGATCT-3'. Indeces were 8 bp long and part of an indexing system developed in-house.

Four independent PCR reactions per sample were prepared using 25% volume of the pre-PCR library each. After 8 cycles of PCR (cycling conditions as per Illumina recommendations) the four reactions were pooled and purified with AmpureXp beads. The final size distribution was determined using a Tapestation 1DK system (Agilent/Lab901). The concentration of each library was determined by Real-time PCR using the Agilent qPCR Library Quantification Kit and a MX3005P instrument (Agilent).

Whole Genome Sequencing was performed on the Illumina HiSeq2000 as 100 bp paired end reads, using v3 clustering and sequencing chemistry. A PhiX control was spiked into the library. We ran 2 lanes of the original library at 21.5 and 23 pM. Then, to “top up” to the required coverage, we ran the library in a multiplex of 16 over 5 lanes at 18 and 18.5 pM.

**Data analysis.** WGS reads were mapped to the human reference genome (GRCh37/d5/hg19) using STAMPY [27] and duplicate reads removed using Picard (http://www.picard.sourceforge.net/). After duplicate reads removal, the mean coverage across the genome was 25.6 x 100 bases covered at 15 x or more. The mean coverage over the 17.1 Mb ROH identified by SNP analysis was 25.9 x with 93.4% of bases covered at 15 x or more. Coverage was calculated with custom scripts and the BEDTOOLS package [33]. Identification of variant sites and alleles was performed with Platypus (written by Andrew Rimmer, Ian Mathieson, Gerton Lunter and Gil McVean: http://www.well.ox.ac.uk/platypus/), which can detect SNPs and short (<50 bp) indels. Briefly, Platypus works by re-aligning reads by putative haplotypes obtained from combining candidate variants, and uses a statistical algorithm to identify the haplotype(s) that best explain the read data, and infer variants and their frequencies.

First, poorly or ambiguously mapped reads are filtered from the data. Platypus requires a minimum mapping quality of 20, which equates to a nominal 1/100 chance of the read being incorrectly mapped. Reads with large numbers of low quality base-calls (>20 bases with quality <10) are also removed. This filtering helps to remove spurious variant candidates caused by poor quality data or reads mapped to difficult regions (e.g. long homopolymers or tandem repeats).

Variant candidates are considered by Platypus if they are seen at least twice in good quality reads. For SNPs, the variant base must be seen at least twice with base-quality > 20. Indel candidates are left-normalised, i.e. the inserted/deleted sequence is reported in the left-most position possible.

Platypus then looks in ~100–200 base windows across the genome, and creates haplotype candidates, based on the list of variants in each window. Each haplotype may contain several variants. A statistical algorithm is used to infer the frequency of each haplotype in the data provided; this algorithm works by re-aligning all the reads to each of the haplotypes, and uses expectation-maximization to estimate haplotype frequencies, and compute a likelihood for each haplotype. Platypus uses these inferred frequencies and the likelihoods to compute a probability for each variant candidate segregating in the data. These probabilities are reported in the final output as a VCF file.

Finally the variants are filtered, to reduce the false-positive rate. First, variants are only called if they have been assigned a sufficiently high posterior probability (the threshold used by Platypus is a phased score of 5). Additional filters are used to remove variants called in low quality reads, or where the variant is only seen on the forward or reverse strand.

We compared the data obtained by the SNP array for V2 with the WGS of V2 and found that 99.83% of the calls were identical, confirming the accuracy of the WGS.

WGS500 Data Freeze 3 (February 2012) includes 213 individual samples. The variant calling was performed as a two step procedure. Initially, variants were called independently for each individual WGS500 sample. The variants from all normal (non-tumour) samples were then merged to generate a union set, containing 26,952,978 unique entries. The second step involved running Platypus on each sample using the variants in the union set as candidates (i.e. as priors). For each variant, the number of occurrences as heterozygous and homozygous in the union set was recorded.

The variants were then processed with a functional annotation pipeline based on the ANNOVAR software package (version of October 2011 [34]). The following ANNOVAR databases (with respect to human genome hg19) were used: ReSeq gene models, dbSNP (Build 132); 1000 genomes allelic frequencies (November 2011); UCSC segment duplication scores; UCSC 46 species conservation scores. Candidate variants were annotated with predictions of functional importance from SIFT [35], PolyPhen2 [36], PhyloP [37] and GERP [38]. We screened known associations to diseases with OMIM (http://www.omim.org/), HGMD Professional (http://www.hgmd.org/), and GeneCards (http://www.genecards.org/).

**Neuropsychological assessments in family.**

Screening of cognitive function was undertaken using the Wechsler Abbreviated Intelligence Scale (WAIS).

**Neuronal cell imaging.**

For immunostaining and histological analysis brains from wild type and β-III spectrin knockout animals were removed and immersion-fixed with either 1 or 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 overnight at 4°C and cryoprotected in 0.1 M sodium phosphate buffer (pH 7.4) containing 30% sucrose. Tissue was embedded in OCT then 16 μm-thick sections cut and mounted onto poly-L-lysine coated slides. Primary antibodies used were mouse anti-MAP2 (Sigma), rabbit anti-tau (DAKO) and rat anti-myelin basic protein (AbD Serotec). Secondary antibodies were cyanine 3 (Cy3)-conjugated goat anti-mouse IgG (Jackson laboratories), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel) and Alexa Fluor 488 –conjugated donkey anti-rat (Invitrogen). For Golgi impregnation brains were removed and immersion-fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 overnight at 4°C and processed as described previously [39]. For cell filling animals were deeply anesthetized with isoethane and sacrificed by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were dissected and postfixed in 1% paraformaldehyde overnight at 4°C. Coronal sections were cut (250 μm-thick) and individual neurons in layer...
2/3 of the prefrontal cortex were visualized with a 20× immersion objective and injected with 0.2 mM Lucifer Yellow (Sigma) and 0.02 mM Alexa FluorAR 568 hydrazide (Invitrogen). Slices were post-fixed and 4% paraformaldehyde overnight at 4°C and were mounted with Vectashield onto 0.13 mm thick borosilicate glass and neurons imaged using the Alexa 568 dye. All images were captured using a Zeiss inverted LSM510 confocal scanning laser microscope and serial stacks used for three-dimensional reconstruction of dendritic arbors using NeuronStudio software (CNIC).

Behaviour tasks in mice

Animals were handled for 1 week and then habituated to the arena (40 cm×40 cm×40 cm) for 5 d before testing. All tests involved a 5 min sample phase followed by a 5 min test phase after a delay of 5 min. Exploratory behaviour was recorded via a WebCam positioned above the testing arena and two researchers blind to genotype scored the investigation of each sample using ANY-maze software (Stoelting). As described previously [16,21] for the novel object preference tasks one object from the sample phase was replaced with a novel object in the test phase; the object-in-place task comprised switching the location of two familiar objects in the test phase; and for the object location task position of one familiar object was changed (Figure 6A–6D). Duplicate copies of familiar objects were used in the test phases to remove any chance of olfactory cues being present. Discrimination ratios were calculated as the time spent exploring the novel or location switched object(s) divided by the total time spent exploring all objects.

Statistical analysis of mouse studies

Statistical analysis was performed using Student’s t-test, two sample assuming unequal variance, apart from analysis of filled pyramidal cells where a two-way ANOVA was used.

Supporting Information

Figure S1 Normal axonal immunostaining in β-III spectrin knockout mice. A. Coronal and sagittal cortical sections from 8-week old WT (+/+ ) and β-III spectrin knockout mice (−/−) immunostained for tau (Bar, 20 μm). B. Coronal sections of cortex and striatum (low and high magnification) stained for MBP (Bar, 20 μm).

(TIF)

Figure S2 Possible disease mechanism of impaired spectrin tetramer formation. α-II shown in yellow, β-II in dark blue, β-III in light blue. Normal tetramers are shown in Black and the mutant dominant tetramers in red bold text and mutant recessive III in light blue. Normal tetramers are shown in Black and the mutant dominant tetramers in red bold text and mutant recessive III in light blue. In the normal there are 4 possible combinations of either α-II/β-II or α-II/β-III. In the SPARCA1 heterozygous carriers tetratomers containing β-II are all normal, but ¼ of the tetramers containing β-III are either absent (if nonsense mediated decay is present) or truncated. These patients are clinically normal illustrating that the tetramers with mutant β-III do not have a dominant effect nor is there haploinsufficiency. In SCA5 the same total number of spectrin tetramers are present as in the heterozygous carriers of the SPARCA1 mutation, but they are clinically affected and the mutation therefore must have a dominant negative effect, rather than be caused by haploinsufficiency. In SPARCA1 all α-II/β-III are non-functional, and in West syndrome, both α-II/β-II and α-II/β-III tetramers are affected resulting in a more severe phenotype.

(TIF)

Table S1 Ataxia genes screened by targeted next generation sequencing. The panel included 117 genes known to cause ataxia in humans, in animal models or were considered likely candidates based on function.

(DOC)

Table S2 Compound Heterozygous variants identified in V2. Compound heterozygous variants identified in V2 filtered as detailed in Material and Methods. a = Wellcome Trust Centre for Human Genetics Whole Genome Sequence data, Freeze 3. b = Exome Variant Server. c = SIFT Probability of being pathogenic; 0 = highest; 1 = lowest. d = Polyphen2 Probability of being pathogenic; 0 = lowest; 1 = highest. e = PhyloP, measures conservation at individual columns of nucleotides. f = PhastCons, is a hidden Markov model-based method that estimates the probability that each nucleotide belongs to a conserved element. g = GERF, Genomic Evolutionary Rate Profiling (GERP) (35 species alignment) conservation score.

(DOC)

Acknowledgments

The authors wish to thank the family for their participation in this study. We also wish to thank Laura Williams and Georgina Bingham, Russell Cairns Unit, for assistance with cognitive assessment; Emma Wood and Matt Nolan for help with the mouse behavioural tests; Richard Källick for supplying anti-tau antibody; and Trudi Gillespie from the IMPACT Imaging facility at the University of Edinburgh for technical assistance with microscopy.

Author Contributions


References

4. Ikeda Y, Dick KA, Weatherspoon MR, Gincel D, Armbrust KR, et al. (2006) 2/3 of the prefrontal cortex were visualized with a 20× immersion objective and injected with 0.2 mM Lucifer Yellow (Sigma) and 0.02 mM Alexa FluorAR 568 hydrazide (Invitrogen). Slices were post-fixed and 4% paraformaldehyde overnight at 4°C and were mounted with Vectashield onto 0.13 mm thick borosilicate glass and neurons imaged using the Alexa 568 dye. All images were captured using a Zeiss inverted LSM510 confocal scanning laser microscope and serial stacks used for three-dimensional reconstruction of dendritic arbors using NeuronStudio software (CNIC).

(DOC)
Cerebellar ataxias: β-III spectrin’s interactions suggest common pathogenic pathways

Emma Perkins, Daumante Suminaite and Mandy Jackson

Centre for Integrative Physiology, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK

Abstract Spino-cerebellar ataxias (SCAs) are a genetically heterogeneous group of disorders all characterised by postural abnormalities, motor deficits and cerebellar degeneration. Animal and in vitro models have revealed β-III spectrin, a cytoskeletal protein present throughout the soma and dendritic tree of cerebellar Purkinje cells, to be required for the maintenance of dendritic architecture and for the trafficking and/or stabilisation of several membrane proteins: ankyrin-R,

This review was presented at the symposium “Mechanisms of cerebellar ataxias and neurodegeneration”, which took place at Ageing and Degeneration: A Physiological Perspective in Edinburgh, UK, 10–11 April 2015.

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cell adhesion molecules, metabotropic glutamate receptor-1 (mGluR1), voltage-gated sodium channels (Na\textsubscript{v}) and glutamate transporters. This scaffold of interactions connects β-III spectrin to a wide variety of proteins implicated in the pathology of many SCAs. Heterozygous mutations in the gene encoding β-III spectrin (SPTBN2) underlie SCA type-5 whereas homozygous mutations cause spectrin associated autosomal recessive ataxia type-1 (SPARCA1), an infantile form of ataxia with cognitive impairment. Loss-of β-III spectrin function appears to underpin cerebellar dysfunction and degeneration in both diseases resulting in thinner dendrites, excessive dendritic protrusion with loss of planarity, reduced resurgent sodium currents and abnormal glutamatergic neurotransmission. The initial physiological consequences are a decrease in spontaneous activity and excessive excitation, likely to be offsetting each other, but eventually hyperexcitability gives rise to dark cell degeneration and reduced cerebellar output. Similar molecular mechanisms have been implicated for SCA1, 2, 3, 7, 13, 14, 19, 22, 27 and 28, highlighting alterations to intrinsic Purkinje cell activity, dendritic architecture and glutamatergic transmission as possible common mechanisms downstream of various loss-of-function primary genetic defects. A key question for future research is whether similar mechanisms underlie progressive cerebellar decline in normal ageing.

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Corresponding author M. Jackson: Centre for Integrative Physiology, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK. Email: mandy.jackson@ed.ac.uk

Abstract figure legend  β-III spectrin is implicated in a number of cellular processes that are essential for maintaining normal Purkinje cell physiology. Disruption to protein trafficking (1), alterations to intrinsic Purkinje cell firing (2), dendritic architecture (3) and glutamatergic neurotransmission (4) have all been identified as common mechanisms across various SCAs.

Introduction

The cerebellum is essential for maintaining postural control and coordination of voluntary muscle movement (Manto, 2008). Purkinje cells, the principal neurons and sole output of the cerebellar cortex, exhibit autonomous high-frequency repetitive firing in addition to receiving input from inhibitory interneurons and two excitatory fibres, climbing and parallel fibres. Purkinje cells integrate the information and transmit timing signals essential for motor coordination in the form of inhibitory inputs to the deep cerebellar nuclei (DCN). The DCN, in turn, communicate with various parts of the nervous system controlling movement. Alterations to Purkinje cell and consequently DCN activity (Shakkottai et al. 2004) are therefore sufficient to induce ataxia, a phenotype characterised by gait disturbances, postural instability and motor incoordination.

Autosomal dominant spinocerebellar ataxias (SCAs), a heterogeneous group of inherited neurodegenerative disorders, are a major cause of cerebellar ataxia. Their prevalence in several populations can be as high as 5–6 in 100,000 (Ruano et al. 2014), similar to that of Huntington’s and motor neuron disease. All SCAs can be characterised by postural abnormalities, progressive motor incoordination and cerebellar degeneration, but a number of subtypes can also be associated with additional neurological features such as cognitive impairment. To date 40 different genomic loci, numbered in order of discovery, have been associated with SCAs and the genes involved, along with the responsible mutations, have been identified for 26 SCA subtypes.

The first genetic defects to be identified as associating with SCA1, 2, 3, 6, 7 and 17 were coding for CAG repeat expansions, leading to proteins with abnormally long poly-glutamine (polyQ) tracts (Orr et al. 1993; Kawaguchi et al. 1994; Imbert et al. 1996; Pulst et al. 1996; Sanpei et al. 1996; David et al. 1997; Zhuchenko et al. 1997; Nakamura et al. 2001). Together they account for more than half of all SCA cases, with SCA3 being the most common (Ruano et al. 2014). Subsequently, non-coding CAG repeats (Holmes et al. 1999; Koob et al. 1999), non-CAG repeat expansions (Matsuura et al. 2000; Sato et al. 2009; Kobayashi et al. 2011) and, more recently, conventional mutations have been found to underlie different SCA subtypes (Table 1). This latter category is ever expanding, due to the advent of whole-exome sequencing, and although conventional mutations are often associated with rarer forms of SCA, they have provided substantial insight into the physiological defects underlying ataxia.

The focus of this review is genetic analyses and use of experimental models to elucidate the pathogenesis of spinocerebellar ataxia type 5 (SCA5). Evidence will be presented demonstrating how changes in Purkinje cell intrinsic excitability, dendritic architecture and synaptic function, observed in mouse models of SCA5,
<table>
<thead>
<tr>
<th>SCA subtype</th>
<th>Gene</th>
<th>Protein</th>
<th>Normal function</th>
<th>Disease mechanism</th>
<th>DNA mutations</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>SCA11</td>
<td>TTBK2</td>
<td>Tau tubulin kinase 2</td>
<td>Protein phosphorylation, cilogenesis</td>
<td>Loss-of-function, DN</td>
<td>FM</td>
<td>Houlden et al. 2007; Goetz et al. 2012</td>
</tr>
<tr>
<td>SCA13</td>
<td>KCNC3</td>
<td>K&lt;sub&gt;3.3&lt;/sub&gt;</td>
<td>Neuronal excitability, K&lt;sup&gt;+&lt;/sup&gt; homeostasis</td>
<td>Loss-of-function, DN</td>
<td>MM</td>
<td>Waters et al. 2006; Irie et al. 2014</td>
</tr>
<tr>
<td>SCA14</td>
<td>PRKCG</td>
<td>Protein kinase C (PKC)</td>
<td>Protein phosphorylation</td>
<td>Unknown</td>
<td>MM, D</td>
<td>Chen et al. 2003</td>
</tr>
<tr>
<td>SCA15/16/29</td>
<td>ITPR1</td>
<td>Inositol 1,4,5-trisphosphate receptor type 1</td>
<td>Calcium homeostasis</td>
<td>Loss-of-function</td>
<td>MM, D</td>
<td>van de Leemput et al. 2007; Iwaki et al. 2008; Huang et al. 2012</td>
</tr>
<tr>
<td>SCA19/22</td>
<td>KCND3</td>
<td>K&lt;sub&gt;4.3&lt;/sub&gt;</td>
<td>Neuronal excitability, K&lt;sup&gt;+&lt;/sup&gt; homeostasis</td>
<td>Loss-of-function, DN</td>
<td>ID, MM</td>
<td>Duarril et al. 2012; Lee et al. 2012</td>
</tr>
<tr>
<td>SCA27/episodic ataxia</td>
<td>FGF14</td>
<td>Fibroblast growth factor 14</td>
<td>Modulation of Na&lt;sup&gt;+&lt;/sup&gt; channels, signal transduction</td>
<td>Loss-of-function</td>
<td>MM, D</td>
<td>van Swieten et al. 2003; Brusse et al. 2006; Shakottai et al. 2009; Choquet et al. 2015</td>
</tr>
<tr>
<td>SCA40</td>
<td>CCDC88C</td>
<td>Coiled-coil domain containing protein 88C</td>
<td>JNK signalling</td>
<td>Gain-of-function</td>
<td>MM</td>
<td>Tsai et al. 2014</td>
</tr>
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</table>

DN, dominant-negative; ID, in-frame deletion; MM, missense mutation; FM, frame-shift mutation; D, large deletion.
have contributed to our understanding of cerebellar dysfunction in SCA5 and how similar physiological defects may be associated with other SCAs.

**Heterozygous mutations in SPTBN2 gene give rise to spinocerebellar ataxia type 5**

The genetic locus for SCA5 was mapped to the centromeric region of the long arm of chromosome 11 (11q13) using a large kindred descended from the paternal grandparents of United States President Abraham Lincoln (Ranum et al. 1994). Later a French (Stevanin et al. 1999) and a German (Burk et al. 2004) pedigree with a similarly mild form of SCA were linked to the same chromosomal region. Mutations were subsequently identified in the SPTBN2 gene encoding β-III spectrin (Fig. 1A; Ikeda et al. 2006), which is found throughout the cell body and dendritic tree of Purkinje cells (Jackson et al. 2001).

The initial symptoms of SCA5 are disturbance of gait, incoordination of limbs, abnormal eye movements and slurred speech. Yet age of onset is variable within families, starting between the second and seventh decade. Typically there is no reduction in lifespan, possibly due to the lack of bulbar paralysis which in other SCAs appears to result in a poorer ability to fight recurrent pneumonia (Zoghbi, 1991) and patients remain ambulatory for several decades. Pathologically severe atrophy of the cerebellum is observed with magnetic resonance imaging (MRI) and autopsy examination shows significant Purkinje cell loss, shrinkage of the molecular layer, mild loss of granular neurons and empty basket fibres (Ikeda et al. 2006).

**Infantile ataxia and cognitive impairment associated with mutations in SPTBN2**

Homozygous mutations in SPTBN2 were recently found in two families with both cerebellar ataxia from childhood and cognitive impairment (Fig. 1B), classifying an allelic condition, spectrin associated autosomal recessive cerebellar ataxia type 1 (SPARCA1) (Lise et al. 2012; Elsayed et al. 2014). A complete loss-of-β-III spectrin function is thus implicated in motor and cognitive deficits from birth. However, a novel heterozygous mutation (R480W) has also been reported in a patient exhibiting infantile onset and global developmental delay (Jacob et al. 2012). It may be that in this case there is an undetected mutation in trans or an environmental modifier resulting in a much earlier and more severe phenotype than

![Figure 1. Structure of β-III spectrin and localisation of mutations](image-url)
other β-III spectrin heterozygous mutations. Alternatively residue R480, within spectrin repeat 2, could be of particular structural importance. Notably, the same heterozygous R480W mutation was recently identified in a child originally given a working diagnosis of ataxia cerebral palsy (Parolin Schnekenberg et al. 2015), strengthening the evidence that mutation R480W is more deleterious than other heterozygous SPTBN2 mutations.

Variability in presentation has similarly been observed for mutations in other SCA-associated loci. Mutations in the inositol 1,4,5-trisphosphate receptor type 1 gene (ITPR1) have been reported in families with late onset SCA15 (van de Leemput et al. 2007), early-onset SCA29 (Iwaki et al. 2008) and sporadic infantile-onset cerebellar ataxia (Huang et al. 2012). Mutations in the genes KCNC3 (Waters et al. 2006; Parolin Schnekenberg et al. 2015) and FGF14 (Coebergh et al. 2014; Planes et al. 2015) have also been associated with variable phenotypes. The molecular reason(s) for differences in timing of onset remain unknown, but the clinical characteristics of patients with early-onset disease are generally non-progressive ataxia, motor developmental delay and mild cognitive deficits. Understanding the molecular mechanisms whereby early-onset cases of ataxia are associated with cognitive impairment could help address whether the cerebellum plays a developmental role in cognition or if the deficits are non-cerebellar in origin.

**Loss-of protein function in cerebellar pathogenesis**

Animal models have proved instrumental in elucidating the pathogenesis of SCA. To date three different SCA5 mouse models have been generated and analysed for signs of motor incoordination and cerebellar degeneration in relation to disrupted β-III spectrin function (Table 2). Two mouse models were created by gene disruption, one by exon trapping (Spnb3−/−; Stankewich et al. 2010) and the other by targeted recombination (β-III−/−; Perkins et al. 2010). The third is a conditional transgenic model which utilises the tetracycline transactivator protein (tTA) under the control of the Purkinje cell specific promoter Pcp2 to specifically drive wild-type or Δ39 β-III spectrin in cerebellar Purkinje cells (Armbrust et al. 2014). All three models exhibit motor impairment but only the β-III−/− mouse model recapitulates the progressive motor deficits and Purkinje cell loss observed in SCA5 patients (Perkins et al. 2010). The β-III−/− mouse model in particular has implicated neuronal dysfunction in the early motor phenotype with gait and coordination deficits evident prior to any cerebellar degeneration. It, together with the identification of the allelic condition SPARCA1, has also provided insights into the molecular dominance in SCA5. Results suggest the disease is due to loss-of-function but not due to β-III spectrin haploinsufficiency as no phenotype is observed in 2-year-old heterozygous (β-III+/−) mice (Clarkson et al. 2010) or elderly heterozygous SPARCA1 carriers (Lise et al. 2012). Instead SCA5 pathogenesis is likely to occur when β-III spectrin function falls below 50% of wild-type level due to interference by mutant protein.

Loss-of protein functions due to dominant-negative effects have also been reported for a number of other SCA subtypes (Table 1). Knock-out animals therefore have the potential to mirror disease phenotypes of autosomal dominant SCAs more faithfully than transgenic models. However, full characterisation for the presence of truncated proteins that could either abrogate loss-of protein function or confer an aberrant function is essential, as is functional analysis to validate models as true knockouts. Creation of representative transgenic models also requires, in order to avoid gene dosage effects on phenotype, detailed information regarding the stability

![Table 2. Molecular, anatomical and behavioural characteristics of mouse lines generated to model SCA5](image_url)
and expression level of mutant proteins and the minimum level of wild-type protein critical for normal function. This is highlighted by work using Drosophila models of SCA5 where progressive neurodegeneration was observed in the Drosophila eye when human β-III spectrin containing either the German (L253P) or American (Δ39) mutation was ectopically overexpressed (Lorenzo et al. 2010). The phenotype, however, was milder in flies hemizygous for L253P spectrin, with these flies expressing significantly lower levels of transgenic protein than flies expressing Δ39 spectrin. The phenotype could be enhanced by increasing L253P spectrin expression through creation of homozygous flies. Similarly posterior paralysis was only observed in larvae expressing a single copy of human Δ39 spectrin when one copy of the endogenous β-spectrin gene was silenced. Hopefully with the advent of new gene targeting technologies (TALENs, Crispr/Cas9) the creation of knock-in models will be greatly facilitated, circumventing caveats surrounding level of transgene expression for loss-of-function models.

Common molecular mechanisms underpinning cerebellar dysfunction in SCAs

Analyses of the different animal models and a number of in vitro studies have implicated various molecular mechanisms in the cerebellar dysfunction associated with SCAs. In particular they converge on alterations to glutamatergic transmission and Purkinje cell excitability, arising from a role for β-III spectrin in membrane protein trafficking, localisation and stabilisation. Disruption to these same physiological processes is evident in models of other SCAs, highlighting the possible convergence of common mechanisms in cerebellar ataxia.

Disruption in membrane protein trafficking, localisation and/or stabilisation. The actin binding domain and C-terminus of β-III spectrin (Fig. 2A) were both shown in a yeast two-hybrid assay to directly bind to Arp1 (Holleran et al. 2001), a subunit of the dynactin complex which mediates the association of vesicular cargo with the microtubule motor dynein (Karki et al. 2000). Further support for β-III spectrin’s role in protein vesicular trafficking is the co-purification from rat brain vesicles with Arp1 and dynein (Holleran et al. 2001) and disruption to axonal transport in flies expressing either the American or German mutant β-III spectrin, with enhancement of these transport abnormalities in dynein and dynactin loss-of-function mutants (Lorenzo et al. 2010). Both β-III spectrin knockout (Spnb3–/– and β-III−/−) mouse lines also exhibit dilatation of endoplasmic reticulum and alterations to Golgi structure indicating an important function of β-III spectrin in the trafficking of membrane proteins (Perkins et al. 2010; Stankevich et al. 2010).

It has been shown that β-III spectrin interacts directly with the carboxy-terminus of EAAT4 (Fig. 2A; Jackson et al. 2001), the glutamate transporter found in Purkinje cell soma and dendrites (Yamada et al. 1996; Dehnes et al. 1998). The interaction stabilises EAAT4 at the plasma membrane, resulting in an increase in cell surface expression and enhanced glutamate uptake (Jackson et al. 2001). In contrast, mutant Δ39 β-III spectrin failed to restrict the lateral mobility of EAAT4 in HEK 293 cells indicating an inability to properly anchor EAAT4 at the plasma membrane (Ikeda et al. 2006). Co-expression of mutant L253P β-III spectrin in HEK 293 cells was also found to disrupt post-Golgi trafficking of EAAT4, with normal cell surface expression only attainable when cells were incubated at a lower temperature (Clarkson et al. 2001).

Figure 2. Defective protein trafficking when β-III spectrin’s scaffold of protein interactions is disrupted

A, schematic diagram depicting identified interacting partners of β-III spectrin: Arp1, ankyrin R, mGluR1 and EAAT4. B and B’, full-length (FL) EAAT4 located at cell membrane and in spine-like protrusions when overexpressed in Neuro2a cells. C and C’, EAAT4 lacking β-III spectrin’s interacting domain within terminal 11 amino acids (EAAT4Δ11) is located peri-nuclearly and in large intracellular vesicles. Scale bar: B and C, 20 μm; B’ and C’, 10 μm.
Table 3. Common Purkinje cell intrinsic activity defects in models of SCA

<table>
<thead>
<tr>
<th>SCA subtype</th>
<th>Physiological deficit</th>
<th>Molecular mechanism</th>
</tr>
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<tbody>
<tr>
<td>SCA1</td>
<td>Reduced intrinsic firing frequency. Irregular plateau potential</td>
<td>Increased (K_\text{v}4.3) surface expression</td>
</tr>
<tr>
<td>SCA2</td>
<td>Reduced intrinsic firing frequency</td>
<td>Increased (Ca^{2+}) release from intracellular stores</td>
</tr>
<tr>
<td>SCA3</td>
<td>Purkinje cells either silent through depolarisation or display faster intrinsic firing rate/burst firing</td>
<td>Increased (K_\text{v}3.3) channel inactivation</td>
</tr>
<tr>
<td>SCA5</td>
<td>Reduced intrinsic firing frequency</td>
<td>Decrease in whole-cell and resurgent sodium current</td>
</tr>
<tr>
<td>SCA13</td>
<td>Reduced intrinsic firing frequency. Broader action potential</td>
<td>Decrease in (K_\text{v}3.3) activity</td>
</tr>
<tr>
<td>SCA27</td>
<td>Reduced intrinsic firing frequency or Purkinje cells silent</td>
<td>Decrease in resurgent sodium current</td>
</tr>
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</table>

Similarly, EAAT4 lacking the terminal 11 amino acids and hence the \(\beta\)-III spectrin binding motif, remains peri-nuclear or in large intracellular vesicular structures when expressed in Neuro2a (Fig. 2C). In contrast full length EAAT4 is present at the cell surface in Neuro2a cells enriched in spine-like protrusions (Fig. 2B). Together these \textit{in vitro} findings support an important role for \(\beta\)-III spectrin in the cellular trafficking and stabilisation of EAAT4 at the plasma membrane. Importantly, reduced EAAT4 levels were observed in young \(\beta\text{-III}^{-/-}\) and \(\text{Spnb}3^{-/-}\) mice with EAAT4 accumulating in the cell soma and dendritic shafts (Perkins et al. 2010; Stankewich et al. 2010; Gao et al. 2011), similar to SCA5 autopsy tissue (Ikeda et al. 2006). Loss of EAAT4 and \(\beta\)-III spectrin prior to onset of symptoms was also reported in a transgenic mouse model of SCA1 that specifically expresses in Purkinje cells human ataxin-1 with a pathological (82) polyglutamine repeat length (ATXN1\textsuperscript{Q82}) (Lin et al. 2000; Serra et al. 2004). More direct evidence that EAAT4 loss is causal in cerebellar dysfunction comes from recent analyses of EAAT4 knockout animals which were found to exhibit motor deficits prior to cerebellar degeneration (unpublished data). No early loss of EAAT4 was observed in the SCA5 transgenic model, but this may be a consequence of the low expression level of \(\Delta39\ \beta\)-III spectrin transgene (Armbrust et al. 2014).

Expression and stability of other membrane proteins have also been reported to be dependent on \(\beta\) spectrin. A decrease in two cell adhesion molecules, neuroglian and Fasciclin II (Fas II), was observed in \textit{Drosophila} lacking presynaptic \(\beta\) spectrin (Pielage et al. 2005) and an altered distribution of Fas II was seen in flies expressing SCA5 mutant spectrin (Lorenzo et al. 2010). Recently \(\beta\text{-III}\) spectrin repeats 14–16 were shown to interact with the metabotropic glutamate receptor mGluR1 and TIRF microscopy revealed wild-type but not mutant \(\Delta39\ \beta\)-III spectrin could increase the stability of mGluR1\textgreek{o}–green fluorescent protein (GFP) at the plasma membrane (Armbrust et al. 2014). The recruitment and maintenance of ankyrin R at the plasma membrane of Purkinje cell dendrites also seems to depend on \(\beta\text{-III}\) spectrin (Clarkson et al. 2014), and further direct evidence for the importance of this interaction in SCA pathogenesis comes from normoblastosis (\textgreek{nb}/\textgreek{nb}) mice, deficient in erythroid ankyrin, which develop abnormal gait, tremor and 50% loss of Purkinje cells by the age of 6 months (Peters et al. 1991).

\(\beta\text{-III}\) spectrin is not believed to be expressed in Bergmann glia, but loss of the glial glutamate transporter GLAST (EAAT1 in humans) was observed in both \(\text{Spnb}3^{-/-}\) (Stankewich et al. 2010) and \(\beta\text{-III}^{-/-}\) (Perkins et al. 2010) mouse lines, indicating an indirect effect on glial membrane protein stability, possibly arising from disruption to cell–cell adhesion and signalling molecules. In \(\beta\text{-III}^{-/-}\) mice the decrease in GLAST has been implicated in the progression of motor deficits (Perkins et al. 2010 and unpublished data) and correlations between decreased GLAST expression and Purkinje cell loss were also reported for transgenic ATXN1\textsuperscript{Q82} SCA1 mice (Cvetanovic, 2015) and mice expressing, only in Bergmann glia, mutant ataxin 7 protein (Custer et al. 2006). Understanding the mechanisms that underpin loss of GLAST will be important as these may highlight potential strategies for mitigating disease progression. There is also evidence that loss of EAAT1 is a primary cause of ataxia with mutations in SLC1A3, the gene encoding excitatory amino acid transporter 1, giving rise to episodic ataxia (Jen et al. 2005; de Vries et al. 2009), further supporting the idea that loss of GLAST is more than a simple consequence of a different primary genetic defect.

Changes to intrinsic Purkinje cell activity. A key component of Purkinje cell output is their intrinsic activity, which has been found in \textit{in vitro} electrophysiological recordings to be altered in various SCA models (Table 3 and Fig. 3). It is governed by specific ion channels and in particular \(Na_{v}1.1\) and 1.6 channels, the two dominant \(Na_{v}\) channels in cerebellar Purkinje neurons, both of which possess a resurgent sodium current (Raman & Bean, 1997; Raman et al. 1997; Khalqi et al. 2010).
Sodium channel dysfunction was observed in the \(\beta^{-}\text{III}^{-/-}\) mouse model of SCA5 prior to cerebellar atrophy with smaller whole-cell and resurgent sodium currents recorded from dissociated Purkinje cells isolated from P16–P20 \(\beta^{-}\text{III}^{-/-}\) mice (Fig. 4; Perkins et al. 2010). This is consistent with the slower rate of Purkinje cell tonic firing observed in cerebellar slices from 3-week-old \(\beta^{-}\text{III}^{-/-}\) mice (Fig. 3B; Perkins et al. 2010).

![Diagram](image)

**Figure 3. Intrinsic activity of Purkinje cells altered in mouse models of SCA**

A, representative trace of a current-clamp in vitro slice recording for a spontaneously firing Purkinje cell. B, slower firing rates compared to controls observed in models of SCA1, 2, 5, 13 and 27. C, quiescent Purkinje cells identified in SCA3 and 27 models, about one-half and 80% of cells, respectively. D, faster tonic firing rate and burst firing observed in remaining SCA3 \(\text{tg}^{-/-}\) Purkinje cells.

![Diagram](image)

**Figure 4. Smaller sodium currents are likely to underpin reduced intrinsic activity**

A, representative current traces from acutely dissociated Purkinje cells elicited with a step depolarisation to \(-30\) mV from a holding potential of \(-90\) mV. B, resurgent sodium currents evoked using a 20 ms step to \(+30\) mV, from a holding potential of \(-90\) mV, followed by repolarisation to \(-30\) mV. Top traces are from wild-type Purkinje cells and bottom traces from \(\beta^{-}\text{III}^{-/-}\) Purkinje cells showing reduced whole cell \(\text{Na}^{+}\) currents and absence of resurgent currents.
and may well be a consequence of decreased Na₃,1.1 and 1.6 stability in the absence of a β-III spectrin/ankyrin R anchor (Clarkson et al. 2014). Functional in vitro analyses of two SCA5-associated mutant β-III spectrin proteins (L253P and R634W) also showed diminished effects in enhancing sodium currents compared to wild-type β-III spectrin with reduced ankyrin R and Na₃ channel levels associated with this effect (Clarkson et al. 2014). Together the data indicate that reduced Purkinje cell intrinsic activity due to a decreased stability of the β-III spectrin/ankyrin-R/Na₃ complex is likely to be a critical component of SCA5 pathogenesis. The heterozygous R480W mutation associated with infantile ataxia was found to have a similar effect to the L253P and R634W mutants (Parolin Schnekenberg et al. 2015) and so additional structural and expression studies are required to resolve whether the change of arginine to tryptophan at residue 480 is more physiologically deleterious than the other heterozygous mutations so far characterised.

A similar decrease in Purkinje cell excitability resulting from sodium channel dysfunction was reported for SCA27 pathogenesis, the genetic causes of which are two loss-of-function mutations, a point mutation (F145S) or a frameshift mutation (Asp163fsX12), in the intracellular fibroblast growth factor 14 (iFGF14) gene (Wang et al. 2002; van Swieten et al. 2005). iFGFs bind directly to the cytoplasmic C-terminal domains of Na₃ channel α subunits, with wild-type FGF14 increasing Na₃ current densities in hippocampal neurones (Lou et al. 2005), whereas peak sodium currents were reduced in cells expressing SCA27 disease associated mutation FGF14/F₁₄₅ˢ (Laezza et al. 2007). It is thought that FGF14 functions as an oligomeric protein and FGF14/F₁₄₅ˢ acts as a dominant negative disrupting the association of wild-type FGF14 and Na₃ channel α subunits. Such a role for loss-of-Nav channel modulation and decreased neuronal excitability in SCA27 pathogenesis is supported by the absence in vitro of spontaneous activity in Purkinje cells both from FGF14-null mice (Shakkottai et al. 2009), which exhibit a very similar phenotype to that of SCA27 patients and following in vivo iFGF14 knock-down studies (Bosch et al. 2015). Reduced Na₃ resurgent sodium current amplitudes and spontaneous firing rates were also observed following acute knockdown of iFGF14 in cultured Purkinje cells (Yan et al. 2014). A key feature of SCA27 pathogenesis appears to be enhanced Na₃ channel inactivation and loss of resurgent current downstream of FGF14 loss-of-function.

K₃ channels are also indispensable for high-frequency intrinsic firing as they exhibit fast activation and deactivation kinetics. Missense mutations (R420H, R423H and F448L) in the gene encoding human K₃,3 (KCNC3) give rise to SCA13 (Waters et al. 2006; Irie et al. 2014). Since all K₃ channels are formed by the assembly of four subunits, K₃,3 channels in SCA13 are likely to consist of WT and mutant subunits with normal function being disrupted in a dominant-negative manner. This is supported by the fact smaller outward currents, broadened action potential wave-forms and a reduced firing frequency are observed in cultured Purkinje cells expressing mouse K₃,3–R424H (Irie et al. 2014), the equivalent of human R423H, similar to in vitro recordings from K₃,3 knockout mice (Hurlock et al. 2008). The resulting delay in Purkinje cell repolarisation is thought to instigate cell death by increasing Ca²⁺ influx through excessive activation of Ca₃ channels.

Mutations giving rise to SCA19/22 were also recently identified in another K₃ subunit, K₃,4.3, and are predicted to reduce cerebellar output, similar to SCA13, as they impair trafficking of the channel to the plasma membrane and/or reduce channel activity (Duarri et al. 2012; Lee et al. 2012). In contrast, accumulation of K₃,4.3 channels at the cell surface was observed in the SCA1 ATXN1⁸¹² mouse model (Houriez et al. 2011). Five-week-old ATXN1⁸¹² mice displayed impaired motor performance and reduced in vitro firing frequencies, with a proportion of cells showing an irregular plateau potential, but no cell atrophy or death at this age. Both the firing frequency and the motor performance were restored by

![Figure 5: Early morphological changes to Purkinje cell dendritic architecture implicated in neuronal dysfunction](image-url)
treatment with DiAP, a potassium channel blocker. The molecular mechanisms underlying the increase in Kv4.3 surface expression and mode of DiAP action are not yet fully understood, although the former is suggested to be linked to reduced Kv4.3 internalisation due to smaller glutamate receptor-mediated postsynaptic currents.

Alterations to Purkinje cell firing prior to signs of neurodegeneration were also observed in in vitro slice recordings for mouse models of SCA3 with 84 glutamine repeats in the ATXN3 gene (Shakkottai et al. 2011) and SCA2 with 127 glutamine repeats in human ataxin-2 cDNA (Hansen et al. 2013). About one half of the SCA3 tg/− Purkinje cells were found to be silent, with a depolarised membrane potential and the others either displayed a faster firing rate than wild-type or exhibited repetitive bursts demonstrating increased excitability (Fig. 3D). Depolarisation block, through increased Kv3 current inactivation, was reported to give rise to the loss of repetitive firing, but how mutant ataxin-3 alters Kv3 channel kinetics is not known. One possibility is that it affects the post-translational modification of potassium channels. In the case of ATXN2Q127 mice a progressive slowing in the firing rate was observed with age but additional analyses are required to determine the molecular mechanisms responsible.

**Altered Purkinje cell dendritic architecture.** Cerebellar output is also influenced by the integration of excitatory and inhibitory inputs that modulate intrinsic Purkinje cell activity (Hauser & Clark, 1997). Since the elaborate monopolar Purkinje cell dendritic tree determines both the number and type of input and how the synaptic signals decay as they propagate towards the soma (Rall, 1977; Hauser et al. 2000; Gulledge et al. 2005), alterations to dendritic morphology will affect Purkinje cell output. In young β-III−/− mice the Purkinje cell dendritic trees were found to be disordered and no longer planar, and dendrites were thinner (Fig. 5; Gao et al. 2011). Membrane properties are affected by dendritic diameter (Rall, 1977) and so aberrant activation of low voltage-gated calcium channels and excessive calcium entry, a potential consequence of thinner dendrites, may contribute to neuronal dysfunction. Loss of planarity prior to cell death can also alter synaptic inputs through interdigitation of neighbouring dendritic trees. Multiple climbing fibre (CF)
innervation can arise via CF transverse branches (Miyazaki & Watanabe, 2011) and disruption to the specificity of granule cell input can occur through ascending axons making synaptic contact with more than one Purkinje cell and/or parallel fibres making additional contacts with the same Purkinje cell (Napper & Harvey, 1988).

Changes to Purkinje cell dendritic architecture were also reported for Purkinje cells in an SCA3 mouse model expressing N-terminally truncated ATXN3Q69 protein (Konno et al. 2014) and for a cellular model of SCA14 (Seki et al. 2009), which is caused by missense mutations in the PRKCG gene encoding protein kinase Cγ (PKCγ). Expression of either mutant S119P or G128D PKCγ in cultured Purkinje cells resulted in reduced dendritic area, dendrite diameter and spine density (Seki et al. 2009). Conventional PKCs require Ca²⁺ for activation and can regulate actin cytoskeleton dynamics through modulation of adducin and recruitment of spectrin to the ends of actin filaments (Matsuoka et al. 1998). Morphological changes could be a common feature in a number of SCAs due to dysregulated Ca²⁺ homeostasis and downstream effects on PKC activity and cytoskeletal dynamics.

Defects in glutamatergic neurotransmission. Purkinje cells, due to the large amount of afferent glutamatergic input they receive from both parallel and climbing fibres through activation of ionotropic AMPA and metabotropic (mGluRs) receptors, are especially vulnerable to glutamate-mediated excitotoxicity and elevations in intracellular calcium (Fig. 6). Aberrant glutamatergic neurotransmission has been observed in two of the SCA5 mouse models. Enhanced parallel fibre-mediated excitatory post-synaptic currents (PF-EPSCs) were recorded from young β-III−/− mice compared to wild-type mice (Fig. 6A and B; Perkins et al. 2010). Although initially the increase in excitability is thought to partially offset the reduced spontaneous activity, the excessive activation of AMPA receptors would appear to be ultimately detrimental, with Purkinje cells from 8-month and older animals found to exhibit dendritic degeneration, undergo dark cell degeneration (Fig. 6C) and have reduced in vivo output (Perkins et al. 2010).

The glutamatergic defect detected in the SCA5 transgenic model is reduced mGluR1 activity following parallel fibre stimulation, due to mislocalisation but not loss of mGluR1 protein (Armbrust et al. 2014). Impairment of mGluR1 signalling was also reported in the ATXN3Q69 mouse model (Konno et al. 2014). A similar loss of mGluR1 signalling may not be detected in the two SCA5 knock-out models as unlike the transgenic animals they both exhibit a loss of EAAT4 protein. EAAT4 modulates the activation of perisynaptic mGluRs, with high EAAT4 expressing Purkinje cells exhibiting very little mGluR activity (Wadiche & Jahr, 2005). Loss of EAAT4 in Spn3−/− and β-III−/−, as well as ATXN1Q82 mice may therefore result in excessive mGluR activation and downstream dysregulated calcium homeostasis. This would be similar to recent findings in a mouse model of SCA28, which is haploinsufficient for Afg3l2 and displays dark cell degeneration of Purkinje cells (Maltecca et al. 2009). Reducing mGluR1 activity was found to decrease Ca²⁺ levels in Afg3l2−/+ Purkinje cells and reverse the ataxic phenotype (Maltecca et al. 2015) indicating attenuating mGluR1 signalling may possess therapeutic promise.

The generation and analyses of various SCA mouse models has revealed possible common physiological deficits downstream of different primary genetic defects. Alterations to intrinsic firing through either direct or indirect effects on ion channels critical for maintaining fast repetitive Purkinje cell firing have been observed in the early stages of cerebellar ataxia in a number of SCA models. The majority of these studies have utilised in vitro slice recordings and so in the future it may be informative to assess the cerebellar output in awake animals. Alterations to Purkinje cell Ca²⁺ homeostasis, in a number of instances arising from altered glutamatergic transmission, are another mechanism common across SCAs which could contribute to dysregulated PKC activity, cytoskeletal alterations, aberrant dendritic architecture and ultimately cell death.

Summary

Cerebellar ataxias can all be characterised by the same clinical features (postural abnormalities, progressive motor incoordination and cerebellar degeneration) highlighting that although the underlying primary genetic defects differ, the downstream molecular mechanisms are likely to converge, with the ultimate effect of altered cerebellar output being common to all. Studies outlined in this review have identified alterations to intrinsic Purkinje cell excitability, dendritic morphology and glutamatergic transmission, arising from disruption to membrane protein trafficking, localisation and stabilisation, as factors pertinent to altered cerebellar output following loss of β-III spectrin function. The necessity for orchestration of protein networks in normal cerebellar physiology is exemplified by the disruption of β-III spectrin function and demonstrates how it is possible that defects in different components of a protein network can instigate the same pathogenic pathway.

Given that motor and cognitive decline are associated with normal ageing, a key question is whether changes to the spectrin submembranous meshwork and key membrane proteins might underpin age-related changes in performance. It has been reported that a progressive increase in α-II spectrin proteolysis (Cai et al. 2012; Hwang et al. 2012), a calcium-dependent process linked to Purkinje cell toxicity (Mansouri et al. 2007), is associated with age. Dilatation of the endoplasmic reticulum and
degeneration of the Golgi apparatus (Dlugos, 2005), reduction in glutamate transporters and functional glutamate uptake associated with mGluR activation (Potier et al. 2010; Brothers et al. 2013; Pereira et al. 2014) as well as changes to the distribution of Na+, channels (Chung et al. 2003) have also all been reported in aged rodents. Dysregulation of glutamatergic neurotransmission and Purkinje cell excitability may therefore be an important feature of age-related cerebellar decline. Similarly cerebellar abnormalities have also been linked to the pathophysiology of Alzheimer’s disease (Sjöbeck & Englund, 2001; Mavroudis et al. 2013), schizophrenia (Andreasen & Prierson, 2008), autism (Courchesne et al. 1994; Palmen et al. 2004; Whitney et al. 2008) and other cognitive and neuropsychiatric disorders (Schmahmann & Sherman, 1998; Konarski et al. 2005; Alalade et al. 2011; Stoodley & Stein 2011). The ongoing challenge for researchers will be to decipher subtle changes in the morphological and molecular integrity of the cerebellar cortex that underpin Purkinje cell dysfunction both in early stages of various neuropsychiatric disorders and in normal ageing.

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### Additional information

#### Competing interests

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#### Author contributions

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Clinical phenotypes of spinocerebellar ataxia type-5 (SCA5) and spectrin-associated autosomal recessive cerebellar ataxia type-1 (SPARCA1) are mirrored in mice lacking β-III spectrin (β-III-/-). One function of β-III spectrin is the stabilization of the Purkinje cell-specific glutamate transporter EAAT4 at the plasma membrane. In β-III-/- mice EAAT4 levels are reduced from an early age. In contrast levels of the predominant cerebellar glutamate transporter GLAST, expressed in Bergmann glia, only fall progressively from 3 months onwards. Here we elucidated the roles of these two glutamate transporters in cerebellar pathogenesis mediated through loss of β-III spectrin function by studying EAAT4 and GLAST knockout mice as well as crosses of both with β-III-/- mice. Our data demonstrate that EAAT4 loss, but not abnormal AMPA receptor composition, in young β-III-/- mice underlies early Purkinje cell hyper-excitability and that subsequent loss of GLAST, superimposed on the earlier deficiency of EAAT4, is responsible for Purkinje cell loss and progression of motor deficits. Yet the loss of GLAST appears to be independent of EAAT4 loss, highlighting that other aspects of Purkinje cell dysfunction underpin the pathogenic loss of GLAST. Finally, our results demonstrate that Purkinje cells in the posterior cerebellum of β-III-/- mice are most susceptible to the combined loss of EAAT4 and GLAST, with degeneration of proximal dendrites, the site of climbing fibre innervation, most pronounced. This highlights the necessity for efficient glutamate clearance from these regions and identifies dysregulation of glutamatergic neurotransmission particularly within the posterior cerebellum as a key mechanism in SCA5 and SPARCA1 pathogenesis.
Introduction

Output from the cerebellar cortex sculpts fine control of motor movements and balance and is derived solely from Purkinje cell neurons, alterations to which result in ataxia. Cerebellar abnormalities may also underlie the pathophysiology in Alzheimer’s disease (1,2), schizophrenia (3), autism (4–6) and other cognitive and neuropsychiatric disorders (7–10).

Mutations in the gene encoding β-III spectrin (SPTBN2) lead to spinocerebellar ataxia type-5 (SCA5) (11) and spectrin-associated autosomal recessive cerebellar ataxia type-1 (SPARCA1) (12), two human neurodegenerative diseases involving gait ataxia and cerebellar atrophy. β-III spectrin is highly expressed in the cerebellum and the phenotype of β-III–/– mutant mice mirrors the clinical phenotypes of SCA5 and SPARCA1 (12,13). Considerable Purkinje cell dysfunction is detectable in young β-III–/– mutant mice prior to cell loss, including increased parallel fibre–Purkinje cell excitatory postsynaptic currents (PF-PC EPSCs) and a 50% reduction in EAA4 protein levels (13). A progressive loss of GLAST is also seen from 3 months of age (13). EAA4 and GLAST are the two principal cerebellar glutamate transporters, and ultrastructural analysis of Purkinje cells from β-III–/– mice revealing dark cell degeneration is consistent with cell death occurring from delayed glutamate-mediated excitotoxicity (13).

We previously demonstrated that β-III spectrin directly interacts with EAA4 and stabilizes high levels of expression at the plasma membrane (14). The early loss of EAA4 in β-III–/– mice is, therefore, almost certainly due to loss of the β-III spectrin anchor. However, the cellular and molecular mechanisms responsible for the delayed loss of GLAST in β-III–/– mice are still to be resolved. The present study uses crosses of ET4–/– and GLAST–/– knockout mice with β-III–/– mice to dissect the relative roles of these glutamate transporters in the pathophysiology of motor deficits. We used these genetic approaches to determine the mechanisms underlying initial hyper-excitability in β-III–/– Purkinje cells (13) and identify which factors previously only correlated with ataxia are directly linked to disease, facilitating the development of effective therapeutic strategies.

Here we demonstrate that loss of EAA4 accounts for the initial hyper-excitability of Purkinje cells lacking β-III spectrin and that loss of GLAST appears to work synergistically to worsen motor deficits. Yet the early loss of EAA4 does not underlie the subsequent loss of GLAST protein. When levels of both EAA4 and GLAST are compromised in β-III–/– mice, the proximal dendrites of Purkinje cells within the posterior cerebellum are the most vulnerable to degeneration. This highlights the importance of efficient glutamate clearance in the vicinity of climbing fibre innervation and identifies this region as an important therapeutic target for SCAs.

Results

EAA4 loss results in Purkinje cell hyper-excitability similar to β-III–/– mice

To discern differences in glutamatergic neurotransmission and investigate the involvement of EAA4 loss in the previously reported β-III–/– Purkinje cell hyper-excitability (13) we measured PF-mediated EPSC amplitudes at increasing stimulus intensities, as widely reported (15–18). This revealed that PF-EPSCs in 6-week old EAA4 knockout (ET4–/–) mice were significantly larger at all stimulus intensities compared to wild type (WT) (P = 1.9 × 10−5 Fig. 1A), similar to β-III–/– mice (13). Moreover, there was no significant difference in amplitude between ET4–/– cells and β-III–/– ET4–/– cells (P = 0.646) indicating EAA4 loss underpins the enhanced PF-PC EPSC amplitudes observed in β-III–/– mice (13).

To ascertain whether the increased EPSC amplitudes were a consequence of pre- or postsynaptic effects paired-pulse facilitation was measured by evoking two presynaptic spikes in close succession (Fig. 1B). This provides an indication of the release probability of the pre-synaptic cell by comparing the postsynaptic response of the second spike to the first spike. No difference in the paired-pulse facilitation ratio was seen between 6-week old WT, ET4–/– and β-III–/–/ET4–/– mice demonstrating changes in presynaptic release probability from parallel fibre terminals are unlikely to underlie the enhanced EPSC amplitudes in ET4–/– and β-III–/–/ET4–/– mice (Fig. 1B). Examination of the decay kinetics revealed the single exponential decay time constant of WT Purkinje cells was similar to the study by Watase et al. (19) for mature Purkinje cells (14.3 ± 3.8 (SD)) and no difference was detected between WT, ET4–/– and β-III–/–/ET4–/– animals (Fig. 1C).

In contrast the decay kinetics in GLAST–/– mice had a single exponential decay time constant 36% longer than WT mice (P = 0.018; data not shown). This is in agreement with other studies (20,21), and further validates our PF-EPSC data.

No difference in AMPA receptor composition in β-III–/– mice

During normal development in the cortex and hippocampus the composition of AMPA receptor changes, with calcium permeable GluA1-containing receptors present early in development being replaced during maturation by calcium impermeable GluA2-containing receptors (22,23). Failure to switch from GluA1 to GluA2, or to edit GluA2 subunits would result in persistent expression of Ca2+–permeable AMPA receptors and EPSCs with larger amplitudes (24–27). Similarly, in the cerebellum it has been reported that by P30 GluA1 is no longer expressed in neurons and is exclusively expressed in Bergmann glia (28). Therefore, to determine whether an abnormal AMPA receptor composition is involved in the enhanced PF-PC EPSC amplitudes observed in Purkinje cells from β-III–/– mice we carried out whole-cell voltage-clamp recordings from 3-week old animals. Inward rectification, a characteristic of receptors lacking GluA2, due to their voltage-dependent block by intracellular polyamines was not observed for either genotype at 3-weeks of age (Fig. 2A and B). In addition, equivalent abundance and cellular distribution of GluA1 immunoreactivity was observed in the molecular layer of β-III–/– animals when compared to wild type animals (Pearson’s correlation coefficient (R = SEM) for GluA1 colocalization: with GFAP P7, WT 0.78 ± 0.55, β-III–/– 0.77 ± 0.54; P14, WT 0.75 ± 0.53, β-III–/– 0.77 ± 0.55; with calbindin P7, WT 0.82 ± 0.58, β-III–/– 0.81 ± 0.57; P14, WT 0.8 ± 0.46, β-III–/– 0.81 ± 0.47; Fig. 2C). Together, these data reveal no difference in the expression profile of GluA1 in β-III–/– animals, with normal edited GluA2-containing receptor compositions in mature β-III–/– Purkinje cells.

Loss of GLAST accentuates motor deficits in young β-III–/– animals

Since a progressive loss of GLAST protein was found to correlate with worsening motor deficits and Purkinje cell loss in β-III–/– animals (13) we investigated whether loss of GLAST is instrumental in disease progression. This was achieved by carrying out the first longitudinal behaviour analysis of GLAST knockout mice using gait analysis, rotarod and an elevated beam task.
There was no difference in hind-limb base width ($P = 0.618$; Fig. 3A), number of slips off the elevated beam ($P = 0.907$; Fig. 3B) or ability to stay on rotarod at 3- and 5-rpm (Fig. 3C) in 6-week old GLAST-/- animals when compared to age-matched WT animals. The main significant deficit observed in young GLAST-/- animals, compared to age matched WT mice, is their ability to remain on the rotarod at 10-rpm ($P = 0.035, 0.046, 0.039, 0.025$, trial 1-4, respectively; Fig. 3C). However, it may be that young GLAST-/- animals are slightly poorer at learning motor tasks than WT animals shown by a potential learning deficit at 3 rpm (Fig. 3C). By 6-months of age GLAST-/- animals have a significantly wider hind-limb base width than when they were 6-weeks old ($P = 0.001$; Fig. 3A) and wider than 6-month old WT animals ($P = 0.004$). They make a greater number of slips on the
elevated beam when 6-months old compared to age-matched WT ($P = 0.003$; Fig. 3B) and to when they were 6-weeks old ($p = 0.002$). Finally by 7.5-months of age they are worse on the rotarod at 3-rpm on day 1 and 2 of testing ($P = 0.035$, 0.008, respectively) and never attain WT performance level, on any day of testing at 10 months of age ($P = 0.02$, 0.0002, 0.005, 0.0002; Fig. 3D).

Moreover, analyses of young $\beta$-III$^{-/-}$/GLAST$^{+/-}$ animals, arising from crossing GLAST$^{-/-}$ animals with $\beta$-III$^{-/-}$ mice, revealed a worse performance on the rotarod at 3-rpm compared to age-matched WT, $\beta$-III$^{-/-}$, GLAST$^{+/-}$ and $\beta$-III$^{-/-}$/ET4$^{+/-}$ animals ($P = 7.73 \times 10^{-5}$, 0.013, 8.55 $\times 10^{-7}$, 0.004; Fig. 4A). Similarly, young $\beta$-III$^{-/-}$/GLAST$^{+/-}$ animals had a significantly wider hindlimb base width ($P = 7.6 \times 10^{-5}$, 1.4 $\times 10^{-4}$, 2.5 $\times 10^{-5}$, $4 \times 10^{-5}$; Fig. 4B) and made more slips on an elevated beam ($P = 1.1 \times 10^{-5}$, 3.8 $\times 10^{-4}$, 8.82 $\times 10^{-7}$, 0.01; Fig. 4C) than WT, $\beta$-III$^{-/-}$, GLAST$^{+/-}$ and $\beta$-III$^{-/-}$/ET4$^{+/-}$ mice, respectively. There was also a significant difference in motor phenotype of young $\beta$-III$^{-/-}$/GLAST$^{+/-}$ animals to both young EAAT4$^{+/-}$ and GLAST$^{+/-}$ animals.

Figure 2. No change in GluA1-containing AMPA receptors in young $\beta$-III$^{-/-}$ Purkinje cells. (A) Superimposed synaptic currents evoked at various holding potentials (-80 to +60 mV, 20 mV increments). (B) Current-voltage relationship for EPSCs recorded from Purkinje cells with spermine in patch pipette. All data are means ± SEM, $N = 3$, $n = 7$ (WT) and 4 ($\beta$-III$^{-/-}$). (C) Midline sagittal cerebellar sections from animals at postnatal day 7 and 14, immunostained with anti-GluA1 antibody. Bar, 50 μm, $N = 3$ for both genotypes.
Finally confocal immunofluorescence microscopy confirmed levels of GLAST protein in young \( \beta \)-III-/- animals were similar to that of GLAST++/− animals (Fig. 4D). Comparison of double mutant \( \beta \)-III-/-/GLAST-/- animals was prevented due to non-Mendelian offspring genotypes arising from genetic crosses and therefore an insufficient number of animals with this genotype were obtained. In contrast no difference was observed between the motor phenotype of young \( \beta \)-III-/- and \( \beta \)-III-/-/ET4-/- mice using rotarod (\( P = 0.999 \); Fig. 4A), hindlimb base width (\( P = 0.963 \); Fig. 4B) and elevated beam (\( P = 0.974 \); Fig. 4C) analyses. Moreover hindlimb base width measurements in 1-year old animals indicated a similar disease progression in \( \beta \)-III-/-/ET4-/- mice (3.1 ± 0.17 cm) to that of \( \beta \)-III-/- animals (3.12 ± 0.11 cm, \( P = 0.919 \)) but an exacerbated phenotype in \( \beta \)-III-/-/GLAST++/− animals (3.63 ± 0.13 cm, \( P = 0.005 \)).

Together, this behavioural data clearly illustrates for the first time that loss of GLAST results in a progressive ataxic phenotype and it provides the first direct evidence that a reduction in GLAST protein levels acts in synergy with loss-of \( \beta \)-III spectrin function to accentuate motor decline, and is not simply an inconsequential side effect.

**Loss of GLAST accelerates Purkinje cell loss in young \( \beta \)-III-/- animals**

To determine whether loss of GLAST is also a key factor in the death of \( \beta \)-III-/- Purkinje cells, we examined the Purkinje cell density in the posterior cerebellum of 3-month old \( \beta \)-III-/-/GLAST++/− mice. The posterior cerebellum was chosen for this temporal analysis as even at 1-year of age in \( \beta \)-III-/- mice we observe no significant loss of Purkinje cells within the anterior cerebellum compared to WT (\( P = 0.996 \); Fig. 5A and B) whereas significant cell death is evident in the posterior cerebellum when compared to WT (\( P = 0.029 \)) and to the anterior cerebellum of \( \beta \)-III-/- mice (\( P = 0.039 \)). Quantification revealed there was cell loss in 3-month old \( \beta \)-III-/-/GLAST++/− mice compared to age-

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**Figure 3.** Progressive motor impairment in GLAST++/− mice. (A) Hind limb base width of 6-week and 6-month old animals. (B) Number of hind-limb slips 6-week and 6-month old mice made when crossing narrow, elevated beam. (C) Latency of 6-week old animals to fall from rotarod at 3-, 5- and 10-rpm. (D) Latency of 7.5- and 10-month old WT and GLAST++ animals to fall from rotarod at 3-rpm. All data are means ± SEM. N = 14 (WT), 14 (young GLAST++), 7 (old GLAST++) in all panels. E, Immunoblot analyses of cerebellar homogenates from 6-week old WT, GLAST++ and GLAST++/− animals.
matched WT animals ($P=0.035$), whereas no significant loss was observed for either $\beta$-III-/- or GLAST-/- animals ($P=0.835$, 0.970; Fig. 5C and D). These results again demonstrate a synergistic effect of reduced GLAST levels and loss-of-$\beta$-III spectrin function resulting in diminished Purkinje cell survival. We see no Purkinje cell loss in either EAAT4-/- or GLAST-/- animals (data not shown).

**Loss of GLAST is not a downstream consequence of early EAAT4 loss**

The mechanism for loss of GLAST protein in $\beta$-III-/- mice is not known but thought to be dependent on Purkinje cell dysfunction; immunofluorescence studies within the cerebellum have so far only shown $\beta$-III spectrin to be expressed in Purkinje cells (13,29). To ascertain whether there is any expression of $\beta$-III spectrin in Bergmann glia, and there is a cell-autonomous effect of $\beta$-III spectrin loss on GLAST protein levels, we carried out semi-quantitative RT-PCR and immunoblot analysis using extracts from primary cerebellar glial cultures and total cerebellar homogenates. No $\beta$-III spectrin transcript (Fig. 6A) or protein (Fig. 6B) was detected in primary cerebellar glial cultures, the purity of which was confirmed by the low level of GLT1 protein expression (Fig. 6B), also an astroglial glutamate transporter but the expression of which is highly-dependent on the co-culturing with neurons (30–32). These results indicate that $\beta$-III spectrin is unlikely to be expressed in Bergmann glia in vivo and demonstrate that in $\beta$-III-/- animals a non-cell autonomous effect most likely underlies loss of GLAST in Bergmann glia.

To determine whether the later loss of GLAST is a downstream consequence of earlier EAAT4 loss from Purkinje cells, possibly mediated by excessive activation of Bergmann glial AMPA receptors (33), we examined GLAST levels in 6-month old ET4-/- mice by immunoblot analyses. No loss was detected compared to WT animals ($P=0.38$; Fig. 6C). Furthermore, when compared to $\beta$-III-/- animals no additional loss of GLAST protein was observed in 6-month old $\beta$-III-/-/ET4-/- mice ($P=0.84$), corroborating the same rate of disease progression observed in $\beta$-III-/- and $\beta$-III-/-/ET4-/- animals (Fig. 4). Since the spectrin-based cytoskeleton has a multitude of functions mediated by interactions with various proteins (34,35) additional downstream consequences of loss-of-$\beta$-III spectrin function in synaptic and structural integrity likely underpin the subsequent loss of GLAST in Bergmann glia.

**Dendritic degeneration in $\beta$-III-/- animals is greater in posterior Purkinje cells with proximal dendrites being the most susceptible**

Finally to investigate further the regional difference in Purkinje cell death in $\beta$-III-/- mice (Fig. 5) we filled, by diffusion from a whole-cell patch pipette, individual Purkinje cells in acute cerebellar sagittal slices with a fluorescent dye. Cells were visualized by confocal microscopy and the dendritic surface area of individual Purkinje cells measured from the anterior (lobules II, III, IV, V) and posterior (VIII, IX, X) lobules (Fig. 7A). This revealed in 6-month old animals a significant difference in the extent of dendritic degeneration between $\beta$-III-/- Purkinje cells from the

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**Figure 4.** Motor decline of $\beta$-III-/- animals accelerated by additional early loss of GLAST. (A) Latency of 6-week-old mice to fall from rotarod at 3-rpm. (B) Hind limb base width of 6-week-old animals. (C) Number of hind limb slips 6-week old mice made when crossing narrow, elevated beam. All data are means ± SEM.
posterior and anterior lobules ($P = 0.05$) and between posterior $\beta$-III$^{-/}$ and WT cells ($P = 0.006$) but not between anterior $\beta$-III$^{-/}$ and WT cells ($P = 0.248$; Fig. 7B and C). However, by 1-year of age, there is a significant difference in the dendritic surface area between $\beta$-III$^{-/}$ and WT cells from anterior lobules (60.9 ± 4.9% of WT, $P = 0.025$, $N = 5, n = 7$ (WT), 9 ($\beta$-III$^{-/}$) and further degeneration of $\beta$-III$^{-/}$ cells from posterior lobules [35.6 ± 6% of WT, $P = 1.5 \times 10^{-5}$, $N = 5, n = 12$ (WT), 9 ($\beta$-III$^{-/}$)].

Images of individually filled Purkinje cells were also used to measure the distance from the cell body to the first branch point and this revealed that in animals >6-months of age this distance was significantly larger in $\beta$-III$^{-/}$ cells from posterior lobules compared to WT cells ($P = 4.0 \times 10^{-7}$) and $\beta$-III$^{-/}$ cells in anterior lobules ($P = 2.4 \times 10^{-7}$). However, there was no difference in distance from the cell body to the first branch point between WT and $\beta$-III$^{-/}$ cells in anterior lobules ($P = 0.988$; Fig. 7B and D). The absence of proximal dendrites in $\beta$-III$^{-/}$ Purkinje cells from posterior lobules was not a developmental defect as they were present in 6-week old $\beta$-III$^{-/}$ mice (Fig. 7E). Moreover, some Purkinje cells (3 out of 9 cells) in anterior lobules exhibited some loss of proximal dendrites at 18-months of age highlighting a progression of proximal dendritic degeneration (Fig. 7F).

Together the data reveal that proximal dendrites are the most susceptible to degeneration, starting within the posterior cerebellum but progressing to the anterior cerebellum, identifying a novel feature of disease pathology (Fig. 8).

Finally, we previously reported that in acute slices the spontaneous activity of $\beta$-III$^{-/}$ Purkinje cells were about half that of WT cells, both at 6-weeks and 6-months of age (13). However, within this study no distinction was made between posterior and anterior lobules. In light of our current observation that the degeneration of the posterior $\beta$-III$^{-/}$ Purkinje cells

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**Figure 5.** Purkinje cell loss in posterior lobules of $\beta$III$^{-/}$ mice accelerated by additional early loss of GLAST. (A) Coronal cerebellar sections from 1-year-old WT and $\beta$III$^{-/}$ mice immunostained with anti-calbindin antibody. (B) Quantification of Purkinje cell density in 1-year-old WT and $\beta$III$^{-/}$ mice. (C) Representative confocal images, from coronal sections, of lobules VIII and IX from 3-month-old mice immunostained with anti-calbindin antibody. (D) Quantification of mean Purkinje cell density in lobules VIII, IX, X and Crus II of hemispheres. All data are means ± SEM, $N = 3$ for each genotype. Bar, 50 μm.
is earlier and greater than that of the anterior Purkinje cells we sought to determine whether there was any variation in the reduction of spontaneous activity in the different populations of β-III-/- Purkinje cells. No significant difference in frequency was observed within the different populations in young or old β-III-/- animals (Fig. 7G) and a similar regularity of firing was observed in all populations (coefficient of variation < 0.2). Nevertheless, it was cells in anterior lobules from 6-week old WT, ET4-/-, βIII-/- and βIII-/-/ET4-/- animals that were found to have the highest spontaneous activity and therefore the closest to that of age-matched WT cells (41.7 ± 4.6 Hz, 0.02 ± 0.003 s).

Discussion

This study conclusively demonstrates that loss of EAAT4 underpins early Purkinje cell hyper-excitability in β-III-/- animals and is the first study to directly show that loss of GLAST accentuates motor deficits and Purkinje cell death, appearing to act in synergy with loss-of β-III spectrin function. Moreover the posterior cerebellum appears to be especially vulnerable to the synergistic effect, likely involving the combined loss of functional EAAT4 and GLAST (Fig. 8). Yet, the observed loss of GLAST in β-III-/- animals does not appear to arise from the early loss of EAAT4 protein.
Figure 7. Proximal dendrites of posterior βIII−/− Purkinje cells most vulnerable to dendritic degeneration. (A) Schematic of sagittal section of cerebellar lobules. (B) Representative confocal images of Purkinje cells filled with Alexa Fluor 568 from anterior (II–V) and posterior (VIII–X) lobules of 6-month and 1-year-old WT and βIII−/− animals. (C) Quantification of dendritic surface area of individual Purkinje cells from 6-month old WT and βIII−/− mice. N = 3, n = 4 (WT), 6 (βIII−/−) for each region. (D) Quantification of distance from Purkinje cell soma to first dendritic branch point in animals > 6-months of age. N = 9, n = 14 (WT), 18 (βIII−/−). (E) Representative confocal images of Purkinje cells filled with Alexa Fluor 568 from posterior (VIII–X) lobules of 6-week old WT and βIII−/− animals. (F) Representative confocal images of Purkinje cells filled with Alexa Fluor 568 from anterior lobules (II–V) of 18-month-old WT and βIII−/− animals. (G) Spontaneous firing frequency and interspike interval of Purkinje cells from young [N = 4, n = 12 (anterior), 19 (posterior)] and old βIII−/− animals [N = 9, n = 28 (anterior), 30 (posterior)]. All data are means ± SEM. Bar, 20 μm.
Loss of β-III spectrin leads to complete loss of functional EAAT4 resulting in Purkinje cell hyper-excitability

Several studies, including our own, have shown EAAT4 levels to be reduced early in various mouse models of ataxia (13–38). For example, we observed loss of EAAT4 in β-III−/− mice, a mouse model of SCAS/SPARCA1, before any Purkinje cell degeneration (13). Similarly reduced EAAT4 levels were observed in young Snb3−/− mice, another mouse model of SCAS, with EAAT4 accumulating in the cell soma and dendritic shafts (38), a feature reported in SCAS autopsy tissue (11). Loss of EAAT4 was also found in a mouse model of spinocerebellar ataxia type 1 (SCA1) prior to any sign of disease (36,39) and EAAT4 levels were found to be decreased in a spontaneous mouse model (staggerer) of ataxia (37). These correlative findings have long suggested that EAAT4 loss may play a key role in ataxia but direct evidence corroborating this association has been lacking.

Here, using EAAT4 knockout mice, we have shown that the greater excitation we observe in young β-III−/− mice are due to loss of EAAT4. A similar increase in peak amplitude of PFPC-EPSCs was observed in young ET4−/− mice as seen in β-III−/− mice (13) and moreover there was no additive effect in β-III−/−/ET4−/− mice when compared to ET4−/− and β-III−/− mice. Together, these data provide compelling evidence that the greater excitation arising from loss-of β-III spectrin function is due to loss of EAAT4 and reduced post-synaptic glutamate uptake. Moreover, despite β-III−/− mice still expressing about 50% of WT EAAT4 protein levels the increase in Purkinje cell excitability is the same for ET4−/− and β-III−/− animals. There is also no difference in motor phenotype between β-III−/− and β-III−/−/ET4−/− mice. It would therefore appear that in the absence of β-III spectrin the remaining EAAT4 protein is not functional. This is an important finding as it suggests that even if levels of EAAT4 could be elevated pharmacologically or through genetic re-introduction it would be unlikely to lead to any therapeutic benefit as without the β-III spectrin anchor the EAAT4 protein would not be correctly targeted or maintained at the plasma membrane.

Our observed change in peak amplitude of PFPC-EPSCs in ET4−/− mice is in contrast to two earlier studies (40,41). However, the animals used in each study were of different ages; Nikkuni et al using 18–22 day old mice (41), Takayusu et al 22–42 day old mice (40), while the present study used 42–46 day old mice. Furthermore, differences in recording conditions (temperature and holding potential) between the three studies also hinder direct comparison and could account for variations in observations. In addition the varying molecular composition of Purkinje cells from different sagittal compartments (42–44) has the possibility of further complicating variability between different data sets.

Loss of β-III spectrin and GLAST act in synergy to exacerbate motor decline

Similar to EAAT4, correlative findings have also suggested loss of GLAST may play a role in the progression of ataxia, but again direct evidence has been lacking. Correlative findings include loss of GLAST and reduced astroglial glutamate uptake when mutant ataxin-7 is solely expressed in Bergmann glia with this mouse displaying ataxia and Purkinje cell death (45). Further correlative evidence comes from conditional ablation of Bergmann glia in adult mice resulting in ataxia and degeneration of Purkinje cell dendrites (46) and loss of GLAST observed in mouse models of SCA1 (47) and SCAS (13) at later stages of disease.

We have carried out the first longitudinal study using GLAST knockout mice and have shown that loss of GLAST gives rise to progressive ataxia. Previously it had been reported that GLAST deficient mice display mild motor deficits, but the age of mice was not reported (19). Here we show a mild motor deficit in young (6-weeks of age) GLAST−/− animals but reveal that the motor deficits are progressive, with old animals performing much worse on all motor tasks. Moreover, through genetic crosses we directly show for the first time that reducing GLAST levels has a severe consequence on the severity of ataxia in β-III−/− animals with young β-III−/−GLAST−/− mice displaying a much earlier decline in motor performance than either β-III−/− or GLAST−/− animals high-lighting a synergistic effect of reduced GLAST and loss-of β-III spectrin function, likely due to a combined loss of EAAT4 and GLAST, the two predominant glutamate transporters in the cerebellum. This conclusion is supported by our analysis of young EAAT4−/−GLAST−/− double knockouts that show the same level of motor dysfunction as β-III−/−GLAST−/− mice (data not shown).

Survival of β-III−/− Purkinje cells in posterior cerebellum dependent on high levels of both EAAT4 and GLAST

The fact loss of Purkinje cells was observed within the posterior cerebellum of young β-III−/−GLAST−/− and old β-III−/− animals indicates that these cells are particularly vulnerable to the synergistic effect of loss of GLAST and β-III spectrin function. GLAST is believed to be present in functional excess throughout the cerebellum (48). In contrast EAAT4 displays a differential pattern of expression within parasagittal bands with Purkinje cells...
in posterior regions possessing higher levels of EAAT4 compared to the anterior regions of the cerebellum (42,44,49). It is therefore Purkinje cells that should normally express high levels of EAAT4 that appear to be more vulnerable to the combined loss of GLAST and β-III spectrin (hence EAAT4). Of note greater glutamate release was shown to occur from climbing fibre terminals in zebrin-positive regions, zones of high EAAT4 expressing Purkinje cells (50). Purkinje cell proximal dendrites, the site of climbing fibre innervation, in the posterior cerebellum may therefore be the most susceptible to degeneration following the combined loss of the two predominant glutamate transporters, EAAT4 and GLAST due to higher levels of glutamate exposure. It is yet to be determined if differences in the functional circuitry between zebrin-positive and negative bands underlie the selective progressive Purkinje cell susceptibility.

There is no significant difference in the reduction of spontaneous firing rate between β-III-/- Purkinje cells with varying extents of cell degeneration. This finding indicates that there is no effect of cell atrophy on reduced voltage-gated sodium channel density following the loss of β-III spectrin function (13,51), in contrast to a reported compensatory effect of neuronal atrophy in a mouse model of SCA1 which restored BK channel density and intrinsic membrane excitability (52).

The molecular mechanism leading to the later but progressive loss of GLAST in β-III-/- mice is unclear. Identifying this mechanism will be paramount in the development of an effective therapy since data from this study directly highlights the critical role loss of GLAST plays in disease progression.

Relevance of posterior cerebellar pathology

The discovery that the posterior cerebellum is the first area affected pathologically in β-III-/- mice provides additional insight into disease pathogenesis and possibly the specific cognitive deficits observed following complete loss of β-III spectrin function (12). Although there are anatomical differences between mice and humans with respect to cerebellar input and involvement of cerebellum in non-motor functions (53), if the human posterior cerebellum is similarly more vulnerable to loss of both EAAT4 and GLAST the functional connectivity between the prefrontal cortex and posterior cerebellum (54,55) would be disrupted in SPARCA1 patients. Of note the posterior cerebellar-prefrontal circuit is involved in cognitive tasks such as attention shifting and verbal working memory tasks (56,57) which appear to be especially impaired in patients with ataxia (58).

In addition the prefrontal cortex and the posterior cerebellum are believed to be significantly affected by ageing (59-61). The discovery that Purkinje cells within the posterior cerebellum are more sensitive to reduced levels of both EAAT4 and GLAST protein may also provide mechanistic insight into age-related decline in motor and cognitive ability. Developing therapeautic strategies to target the posterior cerebellum is likely to prove useful not only in alleviating motor and cognitive deficits associated with inherited ataxias but may also mitigate the effects of normal ageing.

Materials and Methods

Animals

All procedures involved in the generation and analysis of mutant mice were carried out according to the United Kingdom Animals (Scientific Procedures) Act (1986) and other Home Office regulations under specific-pathogen-free conditions. GLAST-/-, ET4-/- and β-III-/- mice, all on a C57BL/6 genetic background, were generated as described previously (13,19,43) and both sexes were used in all experiments. The genotypes of all experimental animals were confirmed by PCR analysis on genomic DNA extracted from ear notch biopsies using ChargeSwitch gDNA tissue kit (Invitrogen, Carlsbad, CA) as described previously (13) or as follows: for ET4-/- mice a common upstream primer (5'-tacctgtgctggaaagattctgg-3') and primers specific for the wild-type allele (5'-agtccaggaaggcctatccctg-3') and the PGR neo cassette in the mutant allele (5'-ggtagcctgcggcaaacg-3') were used for amplification. The 220-bp (from wild-type allele) and 1200-bp (from targeted allele) PCR products were resolved by electrophoresis on a 1.6% w/v agarose gel. For GLAST-/- mice specific primer sets were used for amplification of wild-type allele (5'-aagtccttcgcaacga-3', 5'-aagactctctcagcgtgcc-3') and mutant allele (5'-aatggaggttgagcaaggg-3', 5'-ttcagtggaagtcctgtgg-3'). The 214-bp (from wild-type allele) and 362-bp (from targeted allele) PCR products were resolved by electrophoresis on a 1.6% w/v agarose gel. All knockout mice were viable, although pups from GLAST-/- mice were routinely fostered with CD1 mothers to ensure survival.

Slice electrophysiology

PF-EPSC measurements at a range of stimuli (3-18 V, 200 μs duration) were recorded at room temperature as previously described (13) and the amplitudes and decay time constants (ταu) of PF-evoked EPSCs measured using the NeuroMatic analysis program in IGOR Pro (Wave Metrics, Lake Oswego, OR). Spontaneous action potentials from acute slices were recorded at 30°C ± 2°C as previously described (13). Sagittal cerebellar slices (200 μm) from P16-21 WT and β-III-/- mice were used to determine V/I relationship of PF-EPSCs. Internal solution contained (in mM): 108 Cs +, 2 Na+, 9 NaCl, 9 HEPES, 1.8 EGTA, 1.8 MgCl2, 0.4 NaGTP, 2 MgATP, 63 sucrose and 5 QX-314, adjusted to pH 7.4 with CsOH. Recordings were made at room temperature and picrorotin (50 μM) was added to the ACSF. Stimulus intensity was set to evoke PF-EPSCs of approximately 500 pA at Vh -60 mV (7-42 Hz, 200 μs). Spermine (0.1 mM) was included in the internal solution to replace dialysed endogenous polyamines. V/I relationships for PF-EPSCs in WT and β-III-/- mice were generated by averaging 3 EPSCs at each holding potential (-80 mV to +60mV in 20 mV increments). Series resistances were <15 MΩ and were compensated for by 85%. Data was acquired using pClamp 9 (Molecular Devices, Sunnyvale, CA) and recordings were filtered at 2 kHz and sampled at 10 kHz. Data analysis was carried out in Neuromatic, IGOR Pro (Wave Metrics, Lake Oswego, OR) and using in-house Matlab scripts.

Motor coordination tests

Elevated beam, footprint analysis and rotarod were performed as previously described (13).

Immunohistochemistry

Brains were removed and immersion-fixed with 4% w/v paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 overnight at 4°C and either embedded in paraffin or cryoprotected by immersion in 0.1 M sodium phosphate buffer (pH 7.4) containing 30% w/v sucrose. Paraffin sections (10 μm-thick), mounted on poly-L-lysine-coated slides, were immunostained with anti-GluA1 antibody (Abcam). All other tissues were quick-frozen on
dry-ice, then 30 μm-thick coronal free-floating cerebellar sections immunostained with either anti-calbindin (Swant) or anti-GLAST antibody as described previously (62). All quantification, carried out blind to genotype, involved counting the number of Purkinje cells in the anterior (lobules I-V, simplex and crus I) and posterior cerebellum (lobules VI–IX, crus II and flocculonodular lobe, lobule X) from three sections/animal and the counts averaged. Data were pooled from GLAST+/− and β-III+/−/GLAST−/− animals as no phenotype observed in β-III+/− animals (62). Images were captured with either a Zeiss inverted LSM510 or averaged. Data were pooled from GLAST−/− and nuclear lobe, lobule X) from three sections/animal and the counts carried out blind to genotype, involved counting the number of GLAST antibody as described previously (62). All quantification, of 0.05 or less. N = number of animals and n = number of cells.

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