CEREBELLAR PATHOPHYSIOLOGY IN A MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY

BY

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Abstract

This series of experiments investigated dystrophin localization in the normal cerebellum and examined Purkinje neuron function in normal and dystrophin-deficient mice to better understand the physiological basis for cognitive deficits associated with Duchenne muscular dystrophy (DMD), a common genetic disorder among children. Cognitive impairments are consistently reported in DMD, yet precise mechanisms for their occurrence are unknown. Dystrophin protein, which is absent in DMD, is normally localized to muscles and specific neurons in the brain. Purkinje neurons are rich in dystrophin, specifically in somatic and dendritic membranes. Studies demonstrate perturbed cerebellar function in the absence of dystrophin, suggesting that DMD should be regarded as a cerebellar disorder in addition to being considered a neuromuscular disorder. However, theory and evidence are not generated from overlapping information: research investigating cerebellar involvement in DMD has focused on the vermal region, associated with motor function. The lateral region, implicated in cognition, has not been explicitly examined in DMD. The first experiment revisited the issue of dystrophin distribution in the mouse cerebellum using immunohistochemistry to investigate qualitative and quantitative differences between cerebellar regions. Both regions showed dystrophin localized to Purkinje neuron somatic and dendritic membranes, but dystrophin density was 30% greater in the lateral than the vermal region. The second experiment examined intrinsic electrophysiological properties of vermal and lateral Purkinje neurons from wild-type (WT) mice and from the *mdx* mouse model of DMD which lack dystrophin. Significant differences in action potential firing frequency, regularity, and shape were found between cerebellar regions. Purkinje neurons from *mdx* mouse

cerebellum exhibited membrane hyperpolarization and irregular action potential firing, regardless of region. Spontaneous action potential firing frequency was reduced in Purkinje neurons from lateral cerebellum in *mdx* mice relative to controls, demonstrating that a loss of dystrophin causes a potent dysregulation of Purkinje neuron function in the region associated with cognition. This research extends our understanding of cerebellar pathology in DMD and its potential relevance to cognitive deficits in the disorder. Moreover, this research further supports the role of the cerebellum as a structure important for cognition and contributes to our understanding of dystrophin's role in the brain.

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List of Abbreviations

ANOVA	analysis of variance
BK	big-conductance calcium-activated potassium channel
CB	calbindin
CF	climbing fibres
CNS	central nervous system
CV	coefficient of variation
DCN	deep cerebellar nuclei
DGC	dystrophin-associated glycoprotein complex
DMD	Duchenne muscular dystrophy
DS	dissecting solution
DYS	dystrophin
EBIO	1-ethyl-2-benzimidazolinone
EGTA	ethylene glycol tetraacetic acid
EPSPs	excitatory postsynaptic potentials
FIQ	Full Scale Intelligence Quotient
FOV	field of view
$GABA_A$	γ-aminobutyric acid type A
GL	granule cell layer
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
I_h	hyperpolarized-induced cation current
IQ	intelligence quotient
LTD	long-term depression

mdx	X chromosome-linked muscular dystrophy
MF	mossy fibres
mIPSCs	miniature inhibitory postsynaptic currents
ML	molecular layer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
P	postnatal day
PB	phosphate buffer
PL	Purkinje neuron layer
PET	positron emission tomography
PIQ	performance intelligence quotient
PMCA2	plasma membrane Ca^{++} ATPase subtype 2
PPVT-R	Peabody Picture Vocabulary Test-Revised
RT	room temperature
SEM	standard error of the mean
SK	small-conductance calcium-activated potassium channel
SMA	spinal muscular atrophy
TTX	tetrodotoxin
VIQ	verbal intelligence quotient
WAIS	Wechsler Adult Intelligence Scale
WISC	Wechsler Intelligence Scale for Children
WISC-III	Wechsler Intelligence Scale for Children-III
WISC-R	Wechsler Intelligence Scale for Children-Revised

WRALM	Wide Range Assessment of Learning and Memory
WT	wild-type

Cerebellar Pathophysiology in a Mouse Model of Duchenne Muscular Dystrophy

Chapter 1

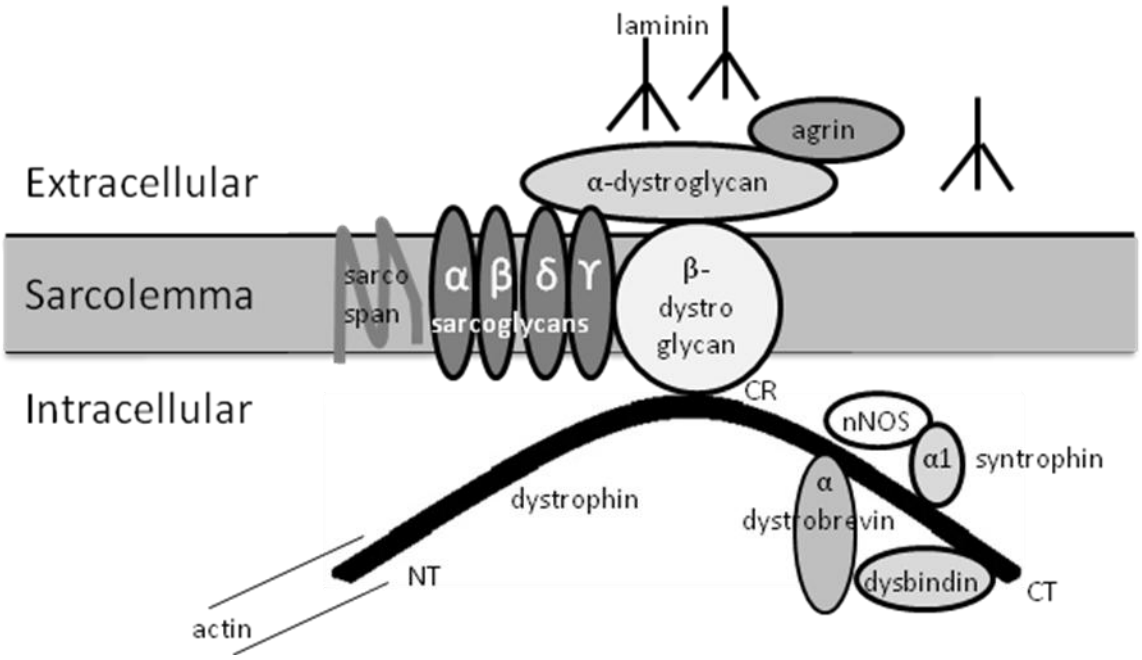
Introduction

1.1. Overview of Duchenne muscular dystrophy and Study Rationale

Duchenne muscular dystrophy (DMD), a common genetic disorder in children, affects approximately one in 3300 boys (Emery, 1991). The X-linked disease occurs as a consequence of a mutation to the *dystrophin* gene, resulting in a lack of expression of the full-length 427 KDa isoform of the large, intracellular protein dystrophin (Hoffman, Brown, & Kunkel, 1987). Initially detected in muscle, dystrophin is part of a large cluster of associated proteins, the cytoskeletal dystrophin-associated glycoprotein complex (DGC), that are linked to form a bridge between the extracellular and intracellular environments (Ervasti & Campbell, 1993) and are essential in maintaining structural integrity of the sarcolemma (muscle membrane) during contraction.

The skeletal muscle DGC is comprised of three sub-complexes and their constituent proteins: the extracellular dystroglycan complex, the transmembrane sarcoglycan-sarcospan complex, and the cytoplasmic complex of dystrophin, dystrobrevins, and syntrophins (Yoshida et al., 1994) (Figure 1.1). The dystroglycan complex consists of both α and β subunits (as reviewed in Allikian & McNally, 2007). The extracellular protein, laminin, binds directly to α-dystroglycan, which in turn binds to β-dystroglycan, thus providing the initial link in the DGC to the extracellular milieu (Ervasti & Campbell, 1993).

Figure 1.1. A schematic representation of the components and associations of the muscle DGC, consisting of the extracellular dystroglycan complex, the transmembrane sarcoglycan-sarcospan complex, and the cytoplasmic complex of dystrophin, dystrobrevins, and syntrophins. NT = N-terminal; CR = cysteine-rich domain; CT = C-terminal.



The sarcoglycan complex of the muscle DGC consists of a family of four transmembrane proteins (α, β, γ, and δ sarcoglycan) that act as a single complex (as reviewed in Allikian & McNally, 2007). Sarcospan binds to the sarcoglycans within the sarcolemma (as reviewed in Allikian & McNally, 2007).

The cytoplasmic proteins of the DGC include dystrobrevins, syntrophins, and dystrophin. Alpha-dystrobrevin directly interacts with cytoplasmic dystrophin and syntrophin (as reviewed in Lapidos, Kakkar, & McNally, 2004). Syntrophins interact directly with dystrophin at its C-terminal as well as with dystrobrevin (as reviewed in Blake, Weir, Newey, & Davies, 2002). Four syntrophin isoforms are localized to varying degrees, in a tissue-specific manner. Inside the sarcolemma, α-syntrophin is found in all

types of skeletal muscle fibres. Syntrophins also bind to numerous other proteins that play key cellular functions, including ion channels and neuronal nitric oxide synthase (nNOS) and, as such, are considered adaptor proteins (Blake et al., 2002; Ervasti, 2007; Perronnet & Vaillend, 2010).

The most-researched component of the complex, dystrophin, has four functional domains: a C-terminal, a cysteine-rich domain, a mid-rod region consisting of 24 spectrin-like repeats, and the N-terminal at which it associates with the intracellular protein actin (Koenig, Monaco, & Kunkel, 1988). The end of the mid-rod domain of dystrophin binds to the transmembrane β-dystroglycan (Blake et al., 2002). Changes to dystrophin expression have a disproportionately large impact on the integrity of the muscle DGC as a whole relative to other DGC components, as changes to localization of many other DGC proteins do not affect dystrophin localization, whereas dystrophin mutations lead to disruptions to the DGC in both humans (Ervasti, Ohlendieck, Kahl, Gaver, & Campbell, 1990) and in mice (Ohlendieck & Campbell, 1991). A more recently identified cytoplasmic DGC member, dysbindin, resides in muscle, where it associates with α-dystrobrevin (Benson, Newey, Martin-Rendon, Hawkes, & Blake, 2001). Unlike other DGC members, in the absence of dystrophin, dysbindin is dramatically upregulated (Benson et al., 2001).

In those with DMD, the absence of dystrophin and downregulation of the remaining DGC components leads to fibre-membrane breakdown, chronic inflammation and muscle-fibre necrosis, with eventual replacement of muscle tissue with connective and fatty tissue. Over time, muscles become progressively weakened, and death occurs

generally in the third decade, due to cardiac and pulmonary complications (Wallace & McNally, 2009).

Physiologically, the mechanisms responsible for fatal muscle pathology associated with DMD are fairly well characterized, as are the protein-protein interactions that constitute the skeletal DGC. There is still no cure for the disease, however, and current treatments focus on alleviating symptoms in hopes of decreasing muscle necrosis and extending life (Bushby et al., 2010). Due to the well-documented muscle pathology, the disease is classically considered neuromuscular in nature.

In his initial description of the disorder, Duchenne (1868) noted decreased non-progressive intellectual abilities in patients with DMD, in addition to the progressive condition affecting muscle. Historically, little attention was paid to cognitive or other neurobehavioural characteristics of those afflicted. This is understandable, given the lethality of the disease that necessitates intensive examination of muscle pathology and the search for potential treatments. However, there is compelling and cumulative evidence to argue for the involvement of the primary central nervous system (CNS) associated with DMD, both at the cellular level and from a clinical perspective. This is so compelling that it has been suggested that DMD should be regarded as a brain disorder in addition to a purely muscle disorder (Hendriksen et al., 2009).

The presence of dystrophin protein in the brain (Lidov, Byers, Watkins, & Kunkel, 1990) provided convincing support for CNS involvement in the disorder. Localization studies revealed that within the CNS, brain dystrophin is most abundant in the cerebellum, where it is restricted to cerebellar Purkinje neurons (Lidov et al., 1990). Decades of research examining cognitive abilities and deficits among those with DMD

suggest a specific neuropsychological profile. The DMD profile is related to deficits specifically in the ability to keep verbal working memory "on-line" and accessible for further information processing, an ability in which the cerebellum has been implicated (as reviewed in Cyrulnik & Hinton, 2008). The cerebellum is traditionally associated in neuroscience references with motor function, motor coordination, and motor learning. However, it is now considered to subserve a broader range of non-motor functions, including cognitive processing (Akshoomoff & Courchesne, 1992) and attention (Allen & Courchesne, 2003). Taking molecular and behavioural data into consideration, it has been argued that DMD should be considered a "cerebellar disorder" (Cyrulnik & Hinton, 2008). Research to date investigating cerebellar dysfunction and DMD using animal models, however, has focused on the vermal cerebellum, the region associated primarily with intact motor function rather than the lateral cerebellum, the region implicated in mental abilities. Thus, this thesis aims to fill a gap in the literature by: 1) re-examining dystrophin localization in the cerebellum, both regionally and subcellularly, and 2) investigating the electrophysiological phenotypes of Purkinje neurons in a mouse model of DMD, in which dystrophin is absent. This series of experiments represents the first in-depth examination of dystrophin and the pathophysiological effects of its absence in Purkinje neurons from the lateral cerebellum, implicated in cognition.

1.2. DMD and Cognitive Function

1.2.1. General Intellectual Functioning

Research examining general intellectual functioning in those with DMD has consistently reported decreased cognitive abilities. An early study (H. J. Cohen, Molnar,

& Taft, 1968) noted an increased prevalence of mental retardation in those with DMD relative to the general population (20.9% versus 3%, respectively) using intelligence quotient (IQ) scores. In that study, IQ scores, in some instances, were estimated from physician, teacher, or parent reports rather than by psychometrically-sound intelligence measures. Using standardized methods of assessing global intellectual functioning, such as the Wechsler Intelligence Scale for Children (WISC; Wechsler, 1949) and Wechsler Adult Intelligence Scale (WAIS; Wechsler, 1939), Prosser, Murphy, and Thompson (1969) noted increased prevalence of mental retardation among those with DMD compared with unaffected siblings, with Full Scale Intelligence Quotient (FIQ) scores, which include scores from both the verbal and performance subtests, approximately one standard deviation below mean sibling FIQ scores in the DMD group. Comparison between sibling controls rules out socioeconomic factors, such as family income and parents' education levels, as primary factors affecting cognitive functioning. Moreover, no significant correlation was found between disease severity and FIQ scores, nor did these scores worsen with age. A. Ogasawara (1989) compared cognitive functioning between DMD sufferers and those with spinal muscular atrophy (SMA), another fatal, progressive neuromuscular disorder. In this group of boys with either DMD or SMA and reared in the same residential school, boys with DMD exhibited decreased FIQ scores relative to those with SMA. Collectively, these reports suggest that impaired intellectual functioning among those with DMD is not secondary to muscle degeneration or the possible environmental consequences and restraints this may place on the child, including reduced educational and social opportunities. As such, these reports point to a primary, organic cause of decreased cognitive abilities in the disorder.

Although the literature documenting impaired cognitive functioning among those with DMD is unequivocal, there has been considerable controversy as to whether there is a selective deficit in verbal intelligence compared to non-verbal intellectual abilities in DMD patients. Numerous studies indicate a more pronounced deficit in regards to the verbal intelligence (VIQ) portion of global intelligence measures as compared to performance intelligence (PIQ) in DMD (Dorman, Hurley, & D'Avignon, 1988; Karagan & Zellweger, 1978; Karagan, 1979; Leibowitz & Dubowitz, 1981; Marsh & Munsat, 1974). Not all studies, however, have detected a difference in VIQ versus PIQ in those with DMD (Black, 1973). To more fully examine the relationship between FIQ, VIQ, and PIQ in DMD, Cotton, Voudouris, and Greenwood (2001) conducted a meta-analysis using compiled data from thirty-two studies from 1960-1999 for which intelligence data were collected. The FIQ, VIQ, and PIQ scores for 1146 males with DMD (age two to twenty-seven years) were subjected to statistical analysis to determine the level of general cognitive impairment, mental retardation prevalence associated with DMD, and the extent to which verbal intelligence is preferentially affected in DMD. On all three intelligence measures, mean scores were normally distributed in the DMD but were shifted approximately one standard deviation below the population mean. Approximately one-third of those with DMD had scores indicative of mental retardation. The mean VIQ-PIQ discrepancy score among DMD males was significantly different from normal at -5.1 (SD=14.4), with a normal distribution of mean scores. These results confirm decades of earlier research demonstrating decreased general intellectual functioning, with increased rates of mental retardation in DMD in arguably the largest, most representative sample examined to date and illustrate decreased verbal relative to non-verbal cognitive ability,

although not to a large degree. The findings also illustrated that intellectual functioning in DMD is variable, as the means on all three estimates of intelligence were all normally distributed.

Although the research points to verbal cognitive function being preferentially impaired in DMD, other possibilities may account for the apparent discrepancy in verbal versus non-verbal abilities. Estimates of non-verbal intelligence using performance subtests on standardized intelligence tests in a group with a recognized motor deficit may introduce artifacts, as subtests comprising the composite PIQ scores are often timed. A score within the typical range is also predicated on intact motor coordination, which could be confounded by motor impairment. Increasing age is positively correlated with increased motor impairments due to the progressive nature of the disease. If discrepancies in PIQ relative to VIQ scores were simply a consequence of impaired motor capabilities and their potential confounding effects on assessments, PIQ scores would be expected to decrease over time in those with DMD.

Several studies have examined the relationship between age and intellectual functioning among those with DMD, garnering ambiguous results. In a longitudinal study, Prosser et al. (1969) found no significant difference between FIQ scores with a group of 29 DMD boys upon serial administrations of the WISC two to six years after initial testing. Moreover, no significant correlation between FIQ or PIQ scores and age was found. A significant positive correlation between VIQ and age was detected, suggesting that verbal intelligence improves over time whereas non-verbal intelligence (as measured by the PIQ), remains static. The results of Black (1973), however, are contradictory to other reports in their findings in a cross-sectional study. Black reported a

significant negative correlation between age and VIQ among DMD boys, suggesting intellectual decline in verbal domains with age. Correlational analyses on FIQ or PIQ scores in this group were not reported in that study. Other cross-sectional studies yielded equally inconsistent results. Leibowitz and Dubowitz (1981) found no correlation between age and FIQ, VIQ or PIQ. Group comparisons between boys with DMD age nine and younger relative to those ten years or older, showed no differences on FIQ, VIQ, or PIQ scores (Dorman et al., 1988), whereas other reports (Miller, Tunnecliffe, & Douglas, 1985) noted that verbal deficits were more pronounced in younger versus older DMD groups.

To examine the possible association between age and FIQ, VIQ, and PIQ in DMD, Cotton, Voudouris, and Greenwood (2005) performed a meta-analysis using the same data set of DMD intelligence scores from their previous study in 2001. Age and disease severity were related, with a higher percentage of DMD males in the older age groups exhibiting more advanced motor impairments. The various age groups in the study, however, did not differ with respect to PIQ scores or FIQ scores. Verbal functioning, as measured by VIQ scores, was significantly higher in the older age groups relative to younger groups. In a similar trajectory, the VIQ-PIQ discrepancy scores were significantly different across the age groups, with the largest discrepancy scores found for the youngest group examined (age nine and under) and smallest in the oldest group (twenty years and older). Presumably, this decrease in discrepancy between the two cognitive domains with age could be explained by an increase in verbal abilities, as non-verbal areas of intelligence, measured by PIQ scores, did not differ with age.

Billard et al. (1992) examined verbal deficits in those with DMD and compared them to those with SMA. In that study, the two groups with similar musculoskeletal disorders did not differ on PIQ scores, but the DMD group had significantly lower VIQ scores relative to the SMA group. These results also indicate that motor impairments do not account for reported discrepancies in estimates of verbal versus performance intelligence in the DMD literature. This observation suggests there is an organic deficit that, unlike motor deficits, does not get progressively worse with age.

1.2.2. Specific Cognitive Deficits in DMD

As evidenced by the normal distribution of IQ scores among those with DMD, boys with DMD exhibit considerable variability in their level of intellectual functioning. It is imperative that we gain a sense of the general cognitive abilities in children with DMD in order to determine the level of CNS involvement in the disorder. Singular dependence on intellectual scores, however, may have limited clinical significance for treating deficits and understanding cognitive abilities and deficits among those with DMD due to intellectual heterogeneity.

Using a neuropsychological approach, which employs specific tests to measure particular cognitive functions, Dorman et al. (1988) administered a battery of 16 tests to a group of DMD adolescents. These tests did not include a motor component and were administered to examine abilities across five cognitive domains: 1) simultaneous processing, 2) sequential processing, 3) auditory analysis, 4) expressive speech, and 5) receptive speech. Based on a cutoff score of one standard deviation below the mean, those with DMD were deficient in three out of four measures assessing sequential

learning with verbal stimuli. This included a test of the ability to recall digits and involved tests of sentence repetition and word order. Deficiencies in simultaneous spatial processing that measured aspects of visuospatial abilities were less apparent, with below-criterion scores on one of four measures. Expressive and receptive speech were not impaired in this sample. Although this study highlights some areas of possible deficiencies in DMD, it did not include a control group.

Whelan (1987) conducted a similar study using neuropsychological assessments and included a control group. Subtests from both the verbal and performance components of the Wechsler Intelligence Scale for Children-Revised (WISC-R; Wechsler, 1974) were administered, as was the Peabody Picture Vocabulary Test-Revised (PPVT-R; Dunn & Dunn, 1981) to assess receptive language and vocabulary, in which the examiner presents a series of pictures, following by a word describing one of the pictures. A child is asked to indicate which picture is best described by the word. The Raven Coloured Matrices test (Raven, 1965) was used to measure general non-verbal cognitive function. Respondents are asked to identify the missing item that completes a presented pattern. Tests of verbal memory (Digit Span from the WISC-R; Sentence Memory Test, Benton, 1965) and non-verbal memory (the Target Test; Reitan & Davis, 1974) were used, and performance on each was compared between those with DMD and SMA against the control group for effects of motor impairment. Despite the large number of assessments, no significant differences were found between the two clinical groups. Within-group comparison of the DMD boys revealed deficits with memory, as they performed more poorly on all tests of immediate verbal and nonverbal memory compared to other cognitive tests.

Other studies have examined specific cognitive functioning in DMD against those with SMA. A. Ogasawara (1989) measured general intellect using the WAIS and WISC between DMD and SMA individuals reared in the same residential home. The DMD group performed significantly more poorly than those with SMA on Digit Span, considered an assessment of immediate verbal memory, where respondents are asked to recite a series of verbally presented digits, although the authors did not report on results from other specific subtests. Billard et al. (1992) also documented specific verbal memory impairments in DMD relative to an SMA group (as a control), namely deficits in story recall. Nearly half of the DMD group also exhibited reading disabilities, whereas the SMA group did not exhibit any such deficits.

Anderson, Routh, and Ionasescu (1988) reported memory deficits among DMD boys compared to age-matched controls. DMD boys were asked to recall the serial positioning of pictures presented from the Peabody Picture Vocabulary Test (Dunn, 1959) and were also administered subtests from the WISC-R, including Digit Span. In addition to deficits in FIQ, VIQ, and PIQ, those with DMD faired much worse on the serial positioning memory task overall relative to controls. Specifically, memory for placement of the first four pictures presented in a series of eight was significantly impaired in the DMD group. Performance on Digit Span (WISC-R) was among the poorest of all subtests given, although a detailed statistical comparison of performance on other subtests is not provided. These findings indicate deficits in both verbal and visual memory in those with DMD.

Other studies using typical control groups have failed to detect deficits in verbal memory. Cotton, Crowe, and Voudouris (1998) compared those with DMD to controls

matched for age, VIQ, and depression to control for its potential effects of cognitive functioning using a battery of neuropsychological tests assessing a number of cognitive domains, including: 1) receptive language, 2) verbal and non-verbal memory, 3) visuospatial skills, 4) attention, and 5) verbal fluency. Verbal memory was not affected in this DMD sample relative to controls matched on numerous variables. However, non-verbal memory was impaired in the DMD group as was attention relative to controls. Visuospatial skills and receptive language were unaffected.

In the first reports of finding specific neuropsychological deficits in DMD compared to unaffected siblings as a control group, Hinton and colleagues put forth an elegant series of studies aimed to elucidate the core neurocognitive deficit(s) associated with DMD. Their first study (Hinton, De Vivo, Nereo, Goldstein, & Stern, 2000) examined 80 boys with DMD, aged six to 16 years, to determine if deficits in certain cognitive domains were associated with general intellectual level or if there is evidence of a specific set of impairments across the IQ spectrum. This research examined performance on numerous neuropsychological assessments with only a minimal motor component, including subtests from the verbal component of the Wechsler Intelligence Scale for Children-III (WISC-III; Wechsler, 1991), specifically Digit Span (recitation of orally presented digits), Comprehension (oral questions answered about social/practical situations), Similarities (orally asked how two items are related or different), and Information (orally answering general information questions). Multiple subtests from the Wide Range Assessment of Learning and Memory (WRALM; Sheslow & Adams, 1990) were also administered, including Story Memory, Picture Memory, Verbal Learning, and Visual Learning. Group mean scores on subtests by the DMD group were compared to

those of 40 unaffected siblings. Performance on subtests for each group was also ranked from worst to best so profiles could be compared between both groups to determine if there was a specific neuropsychological profile associated with DMD. The PPVT-R was used as a proxy measure of general verbal cognitive functioning, as the PPVT-R has a correlation of 0.70 to the WISC-R VIQ. Probands were divided into those scoring above the median on the PPVT-R and those below, and rank order analyses were performed. As a group, probands performed more poorly on the WISC-III Digit Span and Comprehension and on the WRALM Story Memory subtest than their unaffected siblings, and these differences were not accounted for by age. No significant differences were detected among the Similarities or Information subtests from the WISC-R or the Picture Memory, Verbal, or Visual Learning subtests of the WRALM. Rank-order analysis revealed a statistically significant consistency in the rank ordering of performance; a similar pattern was noted for all DMD individuals, as this group performed worse on Digit Span, and in sequence performed better on Comprehension, followed by Similarities, then Information subtests. Rank-order analysis on measures from the WRALM also revealed a significant consistency of ranking, with the worst performance on Story Memory, followed by Picture Memory, Verbal Learning, and Visual Learning subtests. Rank ordering of scores from the sibling group did not reveal a significant ranking consistency, indicating considerable variability in their performance rankings. As such, those with DMD, relative to their unaffected siblings, performed suboptimally on measures of verbal working memory, specifically Digit Span and Story Memory, irrespective of general cognitive functioning. By comparison, memory for pictures and non-verbal stimuli appeared unaffected in those with DMD.

In a subsequent study, Hinton, De Vivo, Nereo, Goldstein, and Stern (2001) used the same sample of DMD individuals and their siblings; DMD participants for whom there were no sibling controls were excluded. This approach enabled use of paired comparisons between each proband and his sibling, thus allowing for a more direct comparison of abilities between those with and without brain dystrophin while controlling for various factors that could affect cognitive abilities (i.e. familial environment, socioeconomic status, etc.). The same eight measures from the WISC-III and WRALM used in the authors' previous study were employed again, with the addition of neuropsychological assessments to gauge functioning in specific cognitive domains: 1) verbal skills, 2) visuospatial skills, 3) attention/memory, and 4) abstract/conceptual skills. Measures of academic achievement in reading, writing, and mathematics from the Woodcock-Johnson Battery (Woodcock & Johnson, 1977) were included. On subtests in the verbal domain, no significant group differences were found. However, paired-comparisons on the individual subtests revealed that DMD boys performed worse on the last two items of the Token Test for Children (DiSimoni, 1978), in which children are asked to follow verbal directions, and these items are thought to rely on an increased auditory load as the test progresses. With respect to visuospatial skills, no significant differences were found in comparisons of overall group means on the collection of subtests or on paired comparisons of individual subtests. The authors also found no significant group differences on tests measuring attention or memory overall. However, paired-comparisons on individual tests indicated that boys with DMD had significantly greater difficulty in Digit Span tests relative to siblings, replicating their previous findings (Hinton et al., 2000). Post hoc analysis revealed that probands had difficulty

particularly with the backward component of that test, in which individuals are asked to recite a list of digits backwards. Those with DMD had lower Comprehension scores on the WISC-R subtest, as per previous findings by the authors. DMD boys also fared worse on all academic-achievement measures, as there were between-group differences on measures of academic achievement and significant differences in paired comparisons on individual academic-achievement tests.

Based on their findings, these researchers (Hinton et al., 2000; 2001) concluded that the neuropsychological profile of DMD is characterized by a selective deficit in verbal working memory skills, as those with DMD consistently did worse on subtests relying on this ability, including Digit Span, Token Test for Children (immediate memory for information processed verbally), and Comprehension (memory for complex verbal information). The authors argued that a decreased ability to keep phonological information within stores of immediate and working memory would be reflected in generalized deficits in assessments of global intellectual functioning. Hinton and colleagues (2000; 2001) proposed that this deficit was particularly noted as depressed scores on the verbal domain tests, as well as decreased overall academic abilities. Impairment in the Digit Span test is commonly cited in neuropsychological examination of DMD individuals (S. W. Anderson et al., 1988; Dorman et al., 1988; Leibowitz & Dubowitz, 1981; A. Ogasawara, 1989; Whelan, 1987). Of note is the finding that boys with DMD showed no impairment in visuospatial skills; this is also consistent with previous studies by other investigators (Cotton et al., 1998; Dorman et al., 1988; Hendriksen & Vles, 2006), suggesting that difficulties are not due to a deficit in visual processing abilities.

The generalized deficits in academic abilities are glaring in light of the relative specificity of differences found using neuropsychological assessments. A decrease in overall academic achievement in those with DMD has been replicated by these authors (Hinton, De Vivo, Fee, Goldstein, & Stern, 2004). Here the authors revised their description of the characteristic cognitive deficit in DMD to one of "verbal immediate memory", or "limited verbal span", rather than their previous description of a specific deficit in verbal working memory. This revision was made according to their finding of deficits in verbal memory when no manipulation of the information is mandated in performing the test involving immediate memory for verbal stimuli rather than working memory *per se*. They further contended that impaired immediate verbal memory is a likely explanation for decreased academic abilities in DMD, noting that an inability to store verbal information during presentation of classroom instruction would negatively impact achievement in all areas of academic ability, including reading, writing, and math. This hypothesis was strengthened by finding a significant contribution from the Digit Span test to scores on all three measures of academic achievement, whereas measures of general cognitive functioning, motor impairment, or parent-rated behavioural problems did not contribute to those measures.

Since this hypothesis of a specific neuropsychological profile characterized by limited verbal span in DMD was published (Cyrulnik & Hinton, 2008; Hinton et al., 2000; 2001; 2004), others have reported deficits in memory related to other types of stimuli and have not fully supported the hypothesis. In a further study elucidating specific memory deficits among those with DMD, Donders and Taneja (2009) used the Children's Memory Scale (M. J. Cohen, 1997) to assess immediate and delayed visual and verbal

memory among boys with DMD and their unaffected siblings. Of these four categories of memory, only delayed memory (not immediate memory) for both stimulus types was affected in DMD relative to unaffected siblings. After controlling for FIQ, however, only differences in verbal delayed recall remained significant. The authors noted that their findings do not support the hypothesis proposed by Hinton and colleagues, and reported that the earlier studies did not examine delayed memory (Hinton et al., 2000; 2001).

The bulk of the literature detailing specific cognitive deficits using neuropsychological measures therefore yields somewhat inconsistent results. Although many report memory deficits, others do not. Moreover, in some cases, memory impairments are restricted to stimuli presented verbally, whereas a minority of reports find deficits in DMD in both verbal and visual memory. The majority, however, do indicate that expressive and receptive language are preserved, as is visuospatial processing. Some of the inconsistencies may be due to the use of various comparison groups (i.e. SMA group, typical control groups matched for various factors, unaffected siblings) as well as the use of different assessments and statistical designs among the studies. Still, there is compelling evidence of a selective deficit in immediate verbal memory in DMD in studies that used carefully controlled comparisons with sound statistical analyses. These reports provide strong support that, in addition to muscle pathology, CNS impairments are a central feature of DMD.

1.3. DMD and CNS pathology

Studies of the cognitive deficits in those with DMD strongly suggest a primary CNS involvement in the disorder. Research examining associated neuropathology,

however, is limited in comparison. The literature documents CNS pathology in DMD in the cerebral cortex, including evidence of gliosis (Dubowitz & Crome, 1969; Jagadha & Becker, 1988), heterotopias (Jagadha & Becker, 1988; Rosman & Kakulas, 1966), neuronal loss (Dubowitz & Crome, 1969; Rosman & Kakulas, 1966) and cortical thickening (Rosman & Kakulas, 1966). Within the cerebellum, a decrease in the number of Purkinje neurons has been reported in DMD at autopsy (Jagadha & Becker, 1988; Rosman & Kakulas, 1966). In the only Golgi-staining study of dendritic morphology in DMD, Jagadha and Becker (1988) noted decreased dendritic length and decreased dendritic branching in brains of all patients examined, although the visual cortex was the only region investigated in that study. The post-mortem studies are limited in number and often consist of a small number of cases of multiple myopathies that are not always restricted to DMD. Magnetic resonance imaging studies revealed no consistent gross brain abnormalities among those with DMD (al-Qudah, Kobayashi, Chuang, Dennis, & Ray, 1990). Although there is some evidence of altered brain structure associated with DMD, the lack of consistent findings in post-mortem and neuroanatomical studies coupled with the noted cognitive deficits suggest that brain anomalies associated with the disorder may be due to more subtle aberrations not detectable by histopathological analysis, such as altered synaptic connectivity or deficiencies in intrinsic electrical properties of neurons lacking dystrophin.

1.4. Dystrophin in the Typical Brain and Dystrophin-deficiency in DMD

The most compelling support for CNS involvement in DMD comes from the finding that dystrophin, the absence of which along the sarcolemma is responsible for

muscle pathology in the disorder, is also present in the typical brain. In the CNS, dystrophin is located within specific populations of neurons that are involved in many higher order functions, such as learning and memory. Dystrophin is localized to cerebellar Purkinje neurons, hippocampal neurons, and pyramidal neurons in the cerebral cortex in the mammalian brain (Huard & Tremblay, 1992; Lidov et al., 1990). CNS dystrophin expression is under the control of different promoters than its muscle counterpart, the so-called M-promoter. The P-promoter regulates Purkinje neuron dystrophin expression, whereas the C-promoter regulates hippocampal and cerebral cortical expression (Blake, Hawkes, Benson, & Beesley, 1999; Gorecki et al., 1992). Truncated dystrophin isoforms are also located in the brain and nervous system, including Dp71, Dp116, Dp140, and Dp260 (as reviewed in Perronnet & Vaillend, 2010). Thus, outside of muscle, the brain is the other main site of *dystrophin* gene expression. Full-length dystrophin[1] is absent, however, in the brains of those with DMD (T. W. Kim, Wu, & Black, 1995; Uchino, Teramoto, Naoe, Miike et al., 1994; Uchino, Teramoto, Naoe, Yoshioka et al., 1994).

Within the CNS, dystrophin expression is highest in the cerebellum, where it is localized as discrete puncta along the somatic and dendritic membranes of Purkinje neurons in both humans (Uchino, Teramoto, Naoe, Miike et al., 1994; Uchino, Teramoto, Naoe, Yoshioka et al., 1994) and mice (Lidov et al., 1990; Lidov, Byers, & Kunkel, 1993; Uchino, Yoshioka et al., 1994). The protein, visualized by electron microscopy, is predominant at postsynaptic sites (Lidov et al., 1990), including dendritic spines (Jancsik & Hajos, 1998). Dystrophin is extensively colocalized with a subset of γ-aminobutyric

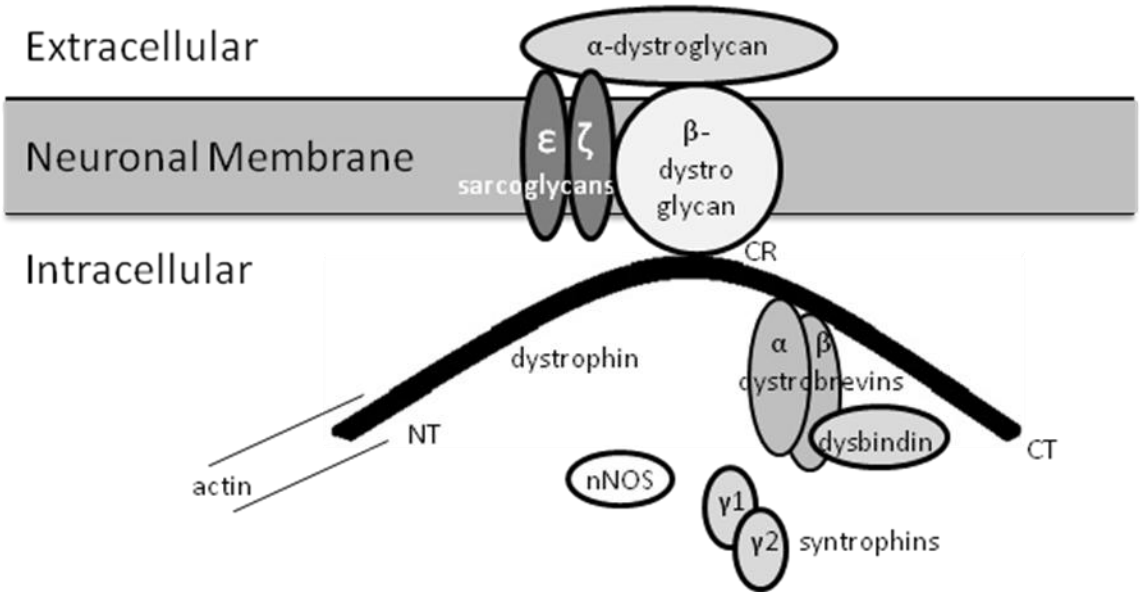
[1]For the sake of clarity and brevity, full-length dystrophin will be simply referred to as "dystrophin". Reference to other isoforms will be stated explicitly.

acid type A ($GABA_A$) receptor clusters in the typical brain, including in the cerebral cortex, hippocampus, and cerebellum (Knuesel et al., 1999).

1.5. Other DGC Members in the CNS

As previously discussed, the cytoskeletal DGC is an integral component of muscle fibres; in the absence of many of these proteins, significant muscle pathology ensues (Ervasti & Campbell, 1991; 1993). In addition to dystrophin, other DGC components have also been identified within the CNS, including cerebellar Purkinje neurons (Figure 1.2). With regard to the extracellular constituents, both α-dystroglycan (Zaccaria, Di Tommaso, Brancaccio, Paggi, & Petrucci, 2001) and β-dystroglycan (Culligan, Glover, Dowling, & Ohlendieck, 2001; Mummery, Sessay, Lai, & Beesley, 1996; Zaccaria et al., 2001) are found in cortical pyramidal neurons. Within the cerebellum, α-dystroglycan is reported in membranes of Purkinje neuron dendrites and somata in a punctate distribution similar to that reported for dystrophin (Smalheiser & Kim, 1995; Tian et al., 1996). Electron microscopy studies show preferential localization of α-dystroglycan to postsynaptic specializations in Purkinje neurons (Zaccaria et al., 2001). Beta-dystroglycan was recently identified in Purkinje neurons and follows a similar punctate distribution as dystrophin in these cells (Anderson, J. E., unpublished data).

Figure 1.2. A schematic representation of the components and associations of the DGC in Purkinje neurons, as currently understood. Unlike that seen in muscle, the syntrophins do not form a strong association with dystrophin in Purkinje neurons. As well, the sarcoglycan complex in Purkinje neurons consists of fewer members than is the case in muscle. The membrane-bound sarcospan, as component of the muscle DGC, is not a member of the DGC in Purkinje neurons. The Purkinje-neuron DGC, however, has an additional dystrobrevin member, β dystrobrevin. NT = N-terminal; CR = cysteine-rich domain; CT = C-terminal.



Within the cerebellum, there are also membrane-spanning portions of the cytoskeletal DGC. Specifically, ε-sarcoglycan has been localized by immunofluorescence to Purkinje neuron somata (Chan et al., 2005) and dendrites (Chan et al., 2005; Yokoi et al., 2011). In addition, ζ-sarcoglycan gene transcripts are found in the CNS, including the cerebellum, although its specific protein localization is poorly understood, as is the composition and inter-protein association of the sarcoglycan complex in brain (Shiga et al., 2006). Unlike the DGC of the muscle-fibre sarcolemma, the transmembrane protein, sarcospan, has not been identified in brain or Purkinje neurons and does not appear to be

a component of the neuronal DGC (Pilgram, Potikanond, Baines, Fradkin, & Noordermeer, 2010; Waite, Tinsley, Locke, & Blake, 2009).

The cytoplasmic dystrobrevins have also been identified within the CNS, including localization in neurons in the cerebral cortex, hippocampus (Blake et al., 1999), and cerebellum (Blake et al., 1999; Grady, Wozniak, Ohlemiller, & Sanes, 2006). In Purkinje neurons, both α-dystrobrevin (Grady et al., 2006) and β-dystrobrevin (Blake et al., 1999) have been identified. Dystrobrevins appear in Purkinje neurons and show a close colocalization with dystrophin, gephryin, and $GABA_A$ receptor clusters; this distribution indicates the presence of dystrobrevins at postsynaptic receptor sites (Grady et al., 2006). Other reports, however, have shown a more diffuse distribution of β-dystrobrevin that is not restricted to Purkinje neurons; in this report, the protein was localized within interneurons in the molecular layer and in granule cells (Blake et al., 1999).

The cytoplasmic component of the DGC called syntrophin has been reported in brain tissue, including observation of both α1 and β2 isoforms of syntrophin within granule cells of the cerebellum (Gorecki, Abdulrazzak, Lukasiuk, & Barnard, 1997). Alternate γ1- and γ2 syntrophin isoforms are present in Purkinje neurons (Alessi et al., 2006). The γ-isoforms do not appear to form a strong association with dystrophin as is the case with α- and β-syntrophin isoforms. Syntrophins, however, have been shown to form a complex with Na^+ channel proteins in studies of membrane extracts from brain tissue (Gee et al., 1998), but this relationship has not been examined in cerebellar tissue specifically. Syntrophin also interacts with inward rectifying K^+ channels from cerebellar tissue, as illustrated using protein purification techniques (Leonoudakis et al., 2004).

As recently documented in muscle, the cytoplasmic protein called dysbindin is also found in brain tissue, where it interacts with β-dystrobrevin (Benson et al., 2001). Dysbindin is present in the cerebellum, including Purkinje neuron somata and dendrites (Benson et al., 2001; Sillitoe, Benson, Blake, & Hawkes, 2003). Its localization, however, is more restricted in Purkinje neurons relative to other DGC members; dysbindin is detected only along the primary apical shaft and does not extend into the finer distal dendrites (Sillitoe et al., 2003).

The literature regarding DGC components in the CNS reveal a complex array of various isoforms that are localized in a tissue- and neuron-specific fashion. There are discrepancies regarding precise localization of these proteins, perhaps due to variation in the quality of immune-detection methods (staining and protein analysis). However, overall the literature suggests a general tendency of DGC components to localize to the postsynaptic membrane of neurons in brain regions that are central to learning and memory functioning, including the hippocampus, cerebral cortex, and cerebellum, and show extensive colocalization with proteins involved in inhibitory synapses. These findings strongly implicate the neuronal DGC in the maintenance of receptors and ion channels. The diversity in DGC form and expression in the typical brain, however, suggests multiple functional roles for DGC proteins (individually and as a complex) in the CNS. The distinctly dense localization of numerous members of the DGC in cerebellar Purkinje neurons, and the extensive presence of dystrophin particularly in these neurons, strongly suggest that DGC members, including dystrophin, play pivotal physiological roles in these neurons, and thus, contribute to proper cerebellar functioning. The following sections provide an overview of the cerebellum as context for the

experiments in this thesis. This overview includes the classical role of the cerebellum as an important component of motor ability, the functional divisions of the cerebellum and more recent perspectives on its functional roles, and a review of the internal circuitry and electrophysiology of its constituent cells, including Purkinje neurons.

1.6. The Cerebellum

1.6.1. Classic View

The first documented experimental evidence for a putative role of the cerebellum in motor functioning came from Rolando in 1809, who noted that cerebellar lesions resulted in motor deficits but preservation of sensory processing abilities (Glickstein & Doron, 2008). In 1824, Fluorens astutely observed that animals with cerebellar lesions were not paralyzed and contended that coordination rather than production of movement was influenced by the cerebellum.

Advances in microscopy during the middle of the 1800s enabled investigators to visualize cellular features of tissue in a way that was not previously possible. Such advances led to the morphological description of Purkinje neurons in unstained tissue by Purkinje in 1837, for whom these cells were named (Glickstein & Doron, 2008). Purkinje described them as “corpuscles” and noted their architecturally orderly arrangement throughout the cerebellum. The extensive dendritic arborizations of Purkinje neurons were not evident, however, as they are not readily obvious in unstained tissue.

It was another 50 years before the unique morphological structure of Purkinje neurons could be appreciated in more detail. One of the most important experimental techniques in neuroscience, still used routinely today, is the silver impregnation method

developed by Golgi in 1873 (Glickstein & Doron, 2008). The exquisite utility of the method lies in the fact that only a small percentage of neurons are stained in their entirety, including soma and processes (Gibb & Kolb, 1998). Thus, the stained neurons are typically observed as single cells, and their processes can be observed in detail without overlapping by other stained cellular processes. Development of this staining procedure (initially from an artifact of histochemical staining), coupled with further advancements in light microscopy, allowed for individual neurons to be visualized with a degree of precision and intensity previously unparalleled. Cajal applied the Golgi method extensively in the late 1880s; his anatomical investigations of multiple brain regions display detailed illustrations. Development of the Golgi technique and its application by Cajal allowed for the characterization of the extensive dendritic arborisation of cerebellar Purkinje neurons for the first time.

In 1891, Luciani applied fine-surgical techniques to investigate cerebellar functioning in vivo, and elaborated on the earlier work of Fluorens who had used animal experimentation (Glickstein & Doron, 2008). Luciani described three features of cerebellar damage associated with various aspects of muscle control: 1) asthenia, or muscle weakness; 2) atonia, or a lack of typical muscle tone; 3) and astasia, or the discontinuity of movement, including tremors. This triad of deficits after cerebellar damage was confirmed in humans by Holmes in 1917 in his clinical investigation of World War I victims of gunshot injuries to the brain (Ito, 2002).

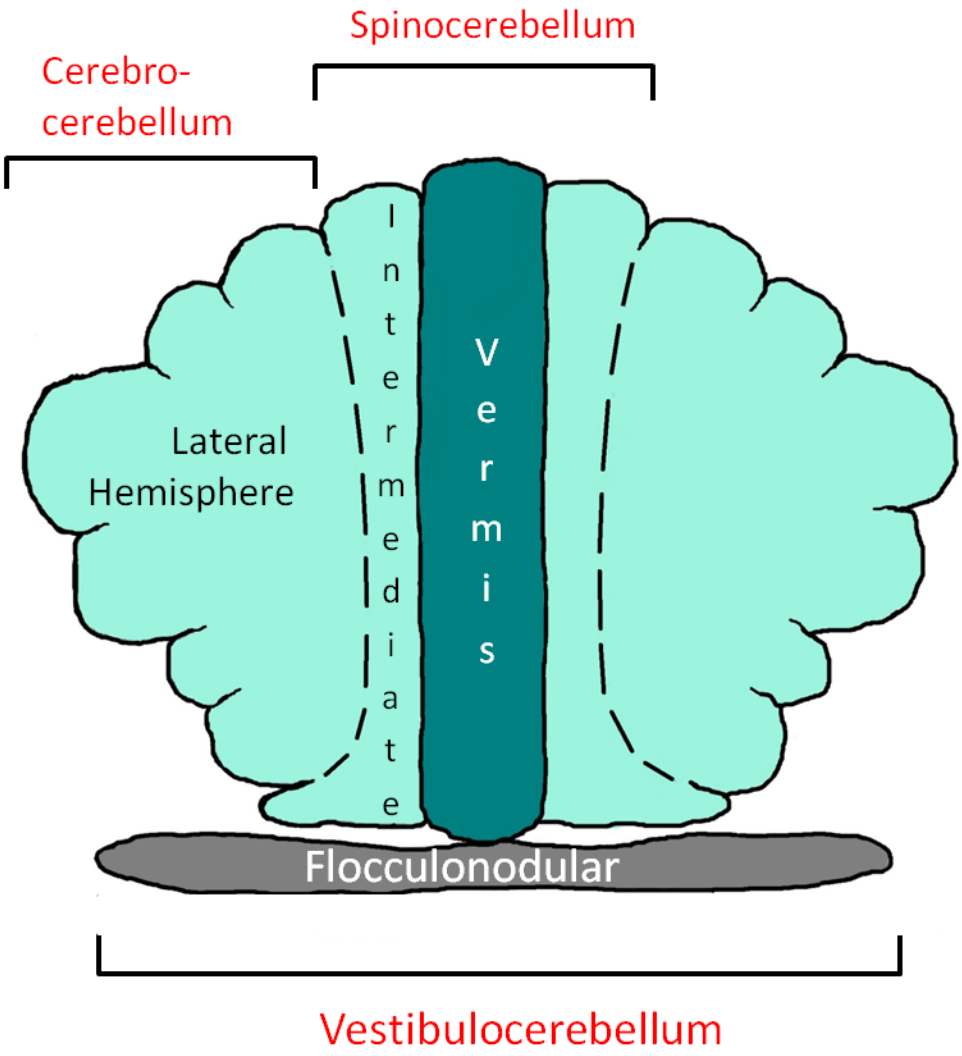
Progress in describing the cerebellar circuitry continued in the 20th Century. Refinement of electrophysiological techniques allowed a group of collaborators in the 1960s to detail the elaborate cerebellar neuronal circuitry. Based on their findings, the

group published a seminal work on the cerebellum, viewing it as a neuronal processing machine based on its intricate circuitry (Eccles, Ito, & Szentágothai, 1967). This monograph provided a jumping-off point for the development of numerous other concepts over the next several decades, in attempts to explain how the unique neuronal circuitry in the cerebellum produces and influences motor behaviour (Albus, 1971; Marr, 1969; Thach, Goodkin, & Keating, 1992).

The cerebellum houses nearly half of the neurons within the brain (Andersen, Korbo, & Pakkenberg, 1992) and receives input from multiple regions of the CNS. The sources of those inputs subserve a broad array of functions and, in turn, the cerebellar neurons project to nearly all portions of the motor system (Thach et al., 1992) as well as non-motor areas of the cerebral cortex (Strick, Dum, & Fiez, 2009). The cerebellum consists of a multineuronal cortical layer, white matter projecting from this layer, and the deep cerebellar nuclei, which are clusters of neurons onto which Purkinje axons synapse. Anatomically, the cerebellum is divided into the flocculonodular lobe, vermis, and lateral cerebellar hemispheres, and functional divisions have been identified based, in part, on these anatomical distinctions (Dow, 1961) (Figure 1.3). The flocculonodular lobe lies on the ventral side of the cerebellum and is the only cerebellar area to bypass the deep cerebellar nuclei in its projections to other areas. It receives information on balance and equilibrium via projections from the nuclei of the vestibular nerve in the brainstem and visual information conveyed from the visual cortex via projections from other nuclei. The projections of the flocculonodular lobe terminate back onto vestibular nuclei within the brainstem, forming a processing loop (Dow, 1961). This region, the phylogenetically oldest region of the cerebellum, is referred to as the “vestibulocerebellum” due to its

pivotal role in processing vestibular information (Dow, 1961; Kandel, Schwartz, & Jessell, 2000).

Figure 1.3. A schematic representation of the anatomical and functional divisions of the cerebellum. Functional divisions are labelled in red.



The vermal cerebellum lies along the midline of the cerebellum and receives multimodal sensory input from the trunk and proximal muscles via spinocerebellar tracts. Projections from the vermis exit the cerebellum via the fastigial deep cerebellar nuclei en route to the brainstem and cortical areas involved in controlling proximal regions of the body, including medial descending pathways (Dow, 1961). Lateral to the vermal region on either side are the intermediate zones. Although not distinct on gross inspection of the cerebellar surface, the intermediate zone receives distinct somatosensory input about distal muscles from spinocerebellar tracts and from the pontine nuclei. In turn, this zone projects to the interposed deep cerebellar nuclei, the projections of which enter the descending lateral corticospinal and rubrospinal tracts that coordinate function of the body musculature (Dow, 1961). Collectively, vermal and intermediate zones are coined "spinocerebellum", as they constitute the only cerebellar regions to receive spinal cord input (Dow, 1961; Kandel et al., 2000).

A third functional subdivision in the cerebellum lies adjacent to the intermediate zones in the most lateral portion of the cerebellar hemispheres. This area, found bilaterally, receives input solely from the cerebral cortex (Dow, 1961), including motor, premotor, prefrontal, and parietal areas (as reviewed in Strick et al., 2009). These cortical inputs travel through the pontine nuclei en route to the cerebellum. Information processed within the lateral cerebellum projects back to motor, premotor, prefrontal, and parietal areas via the dentate deep cerebellar nuclei to thalamic and red nuclei, thus forming a cerebrocerebellar loop (Middleton & Strick, 1997; Strick et al., 2009). As the lateral cerebellar hemispheres receive input exclusively from the cerebral cortex, they are

referred to as the “cerebrocerebellum”, which, phylogenetically, is the newest part of the cerebellum (Dow, 1961; Kandel et al., 2000).

1.6.2. Modern View of the Cerebellum: Relevance to Error Correction and Timing

Although the importance of the cerebellum to motor function is uncontested, precisely how the cerebellum achieves this has been the source of considerable debate. Studies using experimental paradigms with clinical populations have elucidated roles for the cerebellum in both the correction of errors during motor learning and in ensuring accurate timing of both motor and perceptual tasks.

In a now classic experiment, Thach et al. (1992) examined response patterns to throwing a dart at a target before, during, and after visual displacement of the target with prism glasses among individuals with a degenerative disease affecting the inferior olive, a source of excitatory input to cerebellar Purkinje neurons. An individual’s fixation on a target provides a point of aim for the arm throw. When the subject wears prism glasses that bend the optical path to the right, normal individuals will throw the dart to the left of the target. This is in response to the person adjusting their gaze to the left to compensate for the bending of the optic path, as the arm throw follows the direction of gaze. With repeated trials, throws become closer to the target. After removal of the glasses, adjustments made to compensate for the optical displacement persist, despite the gaze now being in-line; as a result, after removing the prism from the optical path, the throws are to the right of the target. Again after practice (this time without the prism glasses), errors in aim are reduced, and throws are on target. Individuals with disease affecting the excitatory input to cerebellar Purkinje neurons fail to adjust their aim in response to the

initial optical displacement with glasses on, and the target is repeatedly to the left, with little improvement in reducing the distance from the target with practice. Upon removal of the glasses, throws from those with cerebellar damage are not far to the right of the target, as is the case for typical individuals, suggesting that the initial adjustments for the prism were never made, and explaining the failure to correct for the visual displacement.

This reduction in error adaptation implicates the cerebellum in providing error correction during the execution of movement. Anatomically, its massive inputs from huge numbers of granule cells are capable of conveying vast amounts of information, and this provides the cerebellum with the neuronal machinery to create a fine representation of incoming information (and adjust to it in near-real time), a feature required for moment-to-moment fine-tuning of motor output (Albus, 1971; Marr, 1969; Thach et al., 1992).

The cerebellum has also been implicated in patterning the timing of both motor and perceptual tasks. Keele and Ivry (1990) examined the motor and perceptual timing abilities of patients with neurological conditions, including cerebellar pathology, cortical pathology, and Parkinson's disease compared to typical controls. In the motor component, subjects tapped their fingers in time to an auditory tone. After the pacing tone was turned off, subjects continued tapping for a specified time, and data were collected regarding the inter-tap variability across trials. In this test, patients with both cerebral and cerebellar pathologies had increased variability in the duration of the inter-tap interval compared to typical controls. During the perceptual component, subjects were presented with two pairs of tones and asked to indicate which one of the two pairs had a longer interval between them. The first pair of tones presented was of a constant duration, and the interval between the second pair of tones was either longer or shorter in duration. The

patients with cerebellar and cortical pathology were impaired on tests of motor timing compared to both typical control subjects and those with Parkinson's disease, ruling out the effect of the diseased basal ganglia to decrease timing abilities. Only patients with cerebellar damage were impaired on the perceptual timing task *and* the motor timing task. Although the increased variability of motor taps could be due to motor impairments in both cortical and cerebellar patient groups, only cerebellar patients were impaired in both timing assessments. To rule out decreased auditory processing abilities as an explanation for the increased variability in the perceptual component, a third task was included in which subjects were presented with pairs of tones as before but were asked to judge which tones were louder. Only cortical patients were impaired on this additional task, suggesting that the altered perceptual timing seen in cerebellar patients was not related to a deficit in general auditory processing or hearing impairments. Based on such findings, the authors hypothesized that the cerebellum attends to and provides temporal information regarding not only the execution of movement but also sensory perception.

1.6.3. The Cerebellum and Mental Skills in Humans

The aforementioned study by Keele and Ivry (1990) was one of the first to reveal non-motor functions that were degraded after cerebellar damage specifically. In addition to studies demonstrating the importance of the cerebellum in perceptual timing, recent advances in functional brain imaging have provided further evidence for the putative role of the cerebellum in non-motor functions. These data are changing the classic view of the cerebellum as a substrate exclusively and intricately involved in motor behaviour, including motor coordination and motor learning; it is now considered to have a broad

role in human behaviour including mediation of cognitive processing (Akshoomoff & Courchesne, 1992). First proposed by Leiner, Leiner, and Dow (1986), the cerebellum's putative role in higher order cognitive functions has since garnered a great deal of interest. Experimental evidence supports cerebellar involvement in mental activity, including the generation of appropriate verbs when presented with nouns (Petersen, Fox, Posner, Mitten, & Raichle, 1989), silent counting (Decety, Sjoholm, Ryding, Stenberg, & Ingvar, 1990), mental imagery (Ryding, Decety, Sjoholm, Stenberg, & Ingvar, 1993) and completion of a pegboard puzzle, with cerebellar activation significantly higher than with simple peg movements (S. G. Kim, Ugurbil, & Strick, 1994).

These studies examining cerebellar activation and cognitive functioning reported activation within the lateral cerebellum and thus support the view that local circuits within the cerebrocerebellum are distinct both anatomically and functionally, from those of the spino- or vestibulocerebellum. In humans, this cerebellar localization of cognitive function is hypothesized to have occurred through evolution, whereby the enlargement of cortical areas has been concomitant with the enlargement in cerebellar circuits associated with the lateral cerebellar cortex, such that cerebellar function has expanded phylogenically to include non-motor, cognitive domains (Leiner et al., 1986).

1.6.4. The Cerebellum and Mental Skills in Mice

In other species, the localization of circuitry within the cerebellum that subserves cognitive versus motor aspects of behaviour is less clear than is currently understood in humans, as is our understanding of abilities in animals that could be deemed "cognitive", in nature (i.e. an organism's mental processing capabilities involving but not limited to

perception, memory, attention, problem solving, self-concept, communication, and associative and spatial learning). Reports on rodents, however, provide evidence that the lateral cerebellum is associated with higher-order information processing outside the parameters of pure motor behaviour. Lesions to the lateral cerebellum in rats result in deficits in spatial learning that do not affect motor abilities (Lalonde & Strazielle, 2003; Joyal et al., 1996; Joyal, Strazielle, & Lalonde, 2001) or impair the acquisition of new memories by observation (Leggio et al., 2000). Lesions to the vermal region, however, lead to impairments in visual guidance to the target platform (Joyal et al., 1996). When lateral cerebellar output is blocked in rats, they are unable to learn tasks involving synchronized activities (Vajnerova, Zhuravin, & Brozek, 2000).

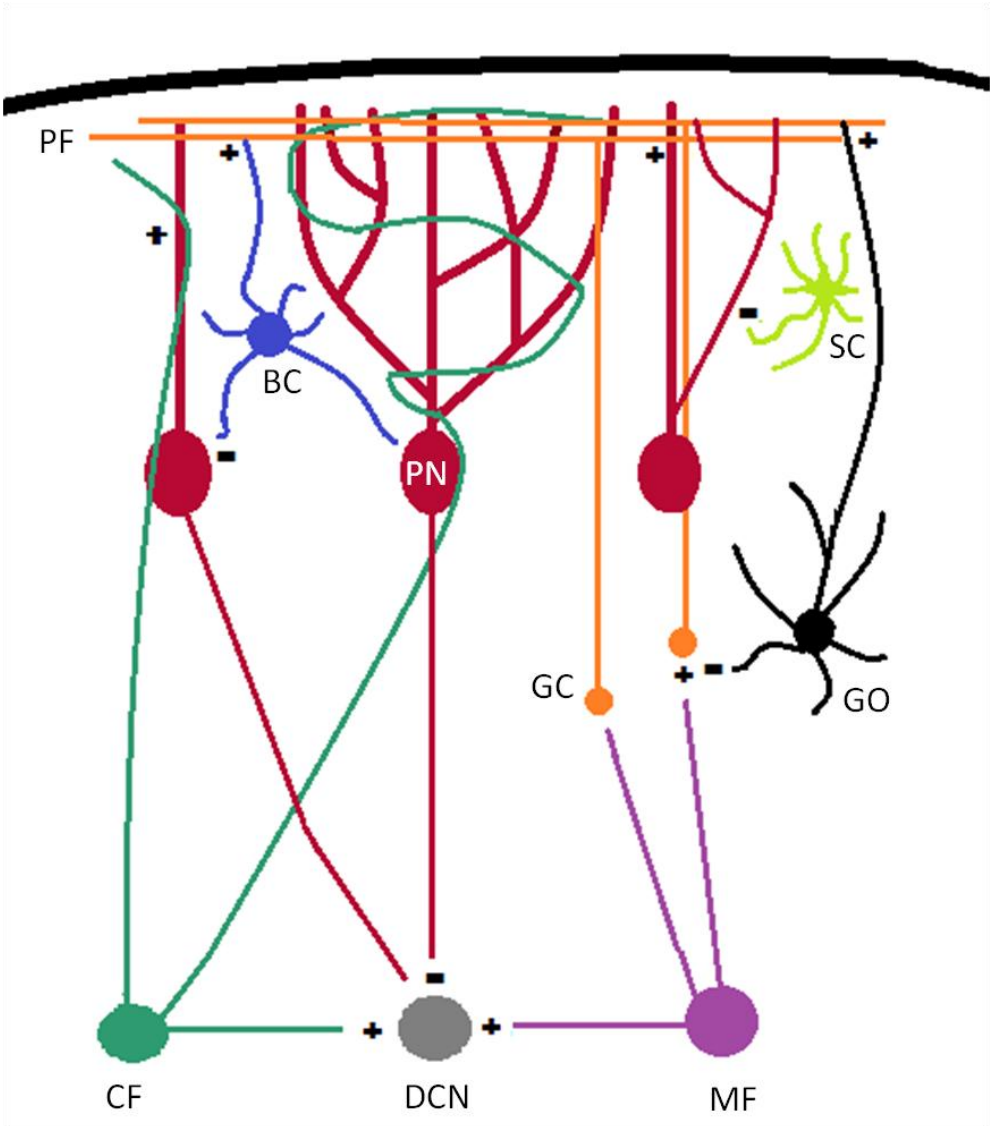
Although the cognitive repertoire differs between man and rodents, both species exhibit clear localization of mental, non-motor activity within the lateral cerebellum. Given that local cerebellar circuitry is preserved across functional subdivisions of the cerebellum (Ito, 2006) and across mammalian species (Hashimoto & Hibi, 2012), lateral cerebellar functioning can be examined in rodent models to investigate the molecular and cellular basis for cognitive function mediated by the normal cerebellum and associated dysfunction in disease states, such as DMD.

1.6.5. Cerebellar Circuitry

The cerebellar cortex (Figure 1.4) consists of three cellular layers comprised of five neuronal types (as reviewed in Hashimoto & Hibi, 2012). The granule cell layer is the deepest cortical layer and is comprised of vast numbers of granule cells, Golgi interneurons, and axons from various inputs, depending on the functional subdivision,

collectively referred to as mossy fibres (MF). A second neuronal layer consists of a monolayer of Purkinje neuron bodies (somata). The outermost layer, the molecular layer, is composed of dendrites of Purkinje neurons, basket and stellate cells, and granule cell axons. The latter are termed parallel fibres due to their arrangement parallel to the parasagittal plane of Purkinje neuron dendrites.

Figure 1.4. A schematic representation of the internal neuronal circuitry of the cerebellum. + = excitatory synapse; – = inhibitory synapse; PF = parallel fibre; BC = basket cell; PN = Purkinje neuron; GC = granule cell; GO = Golgi cell; SC = stellate cell; CF = climbing fibre; DCN = deep cerebellar nuclei; MF = mossy fibre.



This cortical neuronal arrangement is preserved across the subdivisions of the cerebellum (i.e. flocculonodular lobe, vermal and lateral regions); the only difference among the regions is the source of the MF input as well as the deep cerebellar nuclei (DCN) through which the processed signal ultimately leaves the cerebellum (Ito, 2006). Excitatory influences onto Purkinje neurons arise indirectly from the MFs from three sources (i.e. from vestibular nuclei in the vestibulocerebellum or flocculonodular lobe; from the spinal cord via spinocerebellar tracts and reticular formation in the spinocerebellum or vermal and intermediate regions; and from cerebral cortex via the pontine nuclei in the cerebrocerebellum or lateral region). MFs form excitatory synaptic connections with granule cell dendrites, the axons of which extend into the molecular layer in the form of parallel fibres that form excitatory synapses with Purkinje neurons. The excitatory influence of MFs is modified by Golgi interneurons in the granule layer. Axons of Golgi cells form inhibitory synaptic contacts with MF axon terminals and granule cell dendrites in synaptic clusters called cerebellar glomeruli (Hashimoto & Hibi, 2012). These interconnections suppress the excitatory effects of parallel fibres onto Purkinje neurons. The only other source of excitatory projections to Purkinje neurons arises from the inferior olivary nuclei in the medulla. The long axons projecting from the inferior olive, termed climbing fibres (CF), wind around Purkinje neuron somata and their dendrites, and synapse with multiple Purkinje neurons (Eccles, 1967). Basket and stellate cells within the molecular layer exert an inhibitory influence onto Purkinje neurons. Thus, Purkinje neurons are subject to inhibitory influences from cerebellar cortical circuits that collectively modulate the excitatory influences from projections originating outside the cerebellum (Hashimoto & Hibi, 2012).

After integrating multiple inhibitory and excitatory synapses, Purkinje neurons send GABA-ergic inhibitory projections to the DCN (Eccles, 1967) (i.e., the fastigial nucleus in the vermal region, interposed in the intermediate portion, and the dentate nucleus in the lateral hemispheres). Fibres from the DCN project to many regions of the brain, including vestibular nuclei, medial and lateral descending motor pathways, and cerebral cortical association areas (Dow, 1961). In addition to the inhibitory signals from cerebellar Purkinje neurons, neurons in the DCN also receive direct excitatory projections from the two main inputs to the cerebellum, MFs and CFs, via axon collaterals. This direct excitation of the DCN is mediated and dampened by the inhibitory input from Purkinje neurons, the central processors that receive a barrage of both excitatory incoming signals from outside the cerebellum and inhibitory signals from interneurons within the cerebellar cortex. Therefore, Purkinje neurons play a key role in modulating and inhibiting the excitatory influence of the DCN and their broad distribution of terminations in multiple brain regions.

1.6.6. Electrophysiology of Cerebellar Purkinje Neurons

The two main sources of excitation to Purkinje neurons, parallel fibres and CFs, elicit markedly different firing patterns in the target Purkinje neuron in this inhibitory side loop. A single CF elicits strong glutaminergic-excitatory somatic and dendritic synaptic potentials with a single Purkinje neuron, resulting in a pattern of potentials characterized by a strong preliminary action potential, followed by a series of smaller-amplitude action potentials generated at a higher frequency (Eccles, Llinas, & Sasaki, 1964; Llinas & Sugimori, 1980). This firing pattern, propagated by voltage-gated Ca^{++}

channels (Sakurai, 1990), is termed a complex spike and is characteristic of synapses of CFs on Purkinje neurons. Although each Purkinje neuron receives synaptic information from only one CF, a single CF can innervate multiple Purkinje neurons (Eccles, 1967). Incoming sensory or motor stimulation produces only minimal changes in the frequency of CF firing, such that it does not seem to provide an important mechanism for encoding differential aspects of incoming information (Raymond, Lisberger, & Mauk, 1996).

Axons of granule cells, called parallel fibres, on the contrary, do provide a steady rate of glutaminergic excitation on their synaptic targets, Purkinje neurons. Each postsynaptic potential from a parallel fibre, however, is considerably smaller in magnitude relative to those elicited by CF stimulation. Consequently, the input from multiple parallel fibres is summated both temporally and spatially to produce a single action potential in the postsynaptic Purkinje neurons. The frequency of these potentials does change in response to sensory or motor stimuli entering the cerebellum via MFs, unlike potentials evoked by CF stimulation. As such, the firing pattern characteristic of parallel fibre-Purkinje neuron synapses consists of high frequency, small amplitude potentials compared to those seen in complex spikes through CF excitation (as reviewed in Ito, 2000; Ito, 2006).

The disparate discharges of Purkinje neurons elicited from these two excitatory systems have prompted researchers to debate the type of information that is encoded by each system within the cerebellum. Many have argued that the CF input provides the error signal, whereas the MF input provides a fine representation of the movement and its context (Marr, 1969; Raymond et al., 1996; Thach et al., 1992). The enormous number of granule cells that project axons that synapse onto multiple Purkinje neurons as parallel

fibres, makes the MF-granule cell-parallel fibre chain of input an excellent candidate to provide this fine representation within the cerebellum (Raymond et al., 1996).

A unique electrophysiological characteristic of Purkinje neurons is their high level of spontaneous firing in the absence of incoming signals (Hausser et al., 2004). This phenomenon is well-documented and occurs consistently among cells studied after preparation by a variety of methods. The range of methods includes acute slice preparations where inputs are pharmacologically blocked (Hausser & Clark, 1997; McKay & Turner, 2005), acutely dissociated preparations in which the dendritic tree is removed, leaving only the soma (Fry, 2006; Nam & Hockberger, 1997; Raman & Bean, 1997; Raman & Bean, 1999), and in cerebellar cultures (Gruol & Franklin, 1987).

The excitability and action potential firing behaviour of a neuron are dictated by the density, types, and localization of ion channels along the neuronal membrane that allow inward and outward ionic current flow. Within neurons, the ion channels display considerable diversity in the pattern of subcellular localization, density, and the mechanisms of channel kinetics and gating (for a thorough review, see Vacher, Mohapatra, & Trimmer, 2008). Purkinje neurons are no exception; they possess a wide complement of ion channels and accompanying currents that shape action potential properties. For example, Purkinje neurons possess three classes of Na^+ currents, each with unique voltage-dependencies and gating properties: transient, persistent, and resurgent Na^+ current. First described in detail in the giant squid axon by Hodgkin and Huxley (Hodgkin & Huxley, 1952), a conventional large, inward tetrodotoxin (TTX)-sensitive Na^+ current activates at a particular threshold and contributes substantially to the rising phase of an action potential (“transient current”). This current activates and

inactivates rapidly, and, thus, also contributes to sustained tonic spontaneous firing in the absence of synaptic inputs (Raman & Bean, 1997). A persistent Na^+ current becomes activated at a subthreshold membrane potential and distinct from transient Na^+ current, remains activated even after long periods of depolarization (Kay, Sugimori, & Llinas, 1998). Another important subthreshold Na^+ current, the resurgent Na^+ current, activates after large depolarizations of the membrane potential and contributes Na^+ current during membrane repolarization, thus allowing for the rapid production of successive action potentials (Raman & Bean, 1997).

Multiple studies have demonstrated the importance of Na^+ current in contributing to the properties of action potential firing. Application of TTX, which blocks Na^+ channel activation, abolishes somatic firing (Raman & Bean, 1999). TTX-sensitive resurgent Na^+ current contributes to Purkinje neuron excitability and spontaneous activity. In *med* mice, a mutation in the SCN8A gene encoding the Nav1.6 Na^+ channel α subunit results in diminished resurgent Na^+ current, implicating this isoform of the Na^+ channel in resurgent current generation (Raman, Sprunger, Meisler, & Bean, 1997). As a result, spontaneous activity is diminished (Raman et al., 1997). In mice where Nav1.6-deficiency is secondary to mutations in the gene for fibroblast growth factor 14, Purkinje neurons are incapable of high-frequency firing, regardless of stimulus intensity (Shakkottai et al., 2009). This observation highlights the importance of this channel subunit in firing ability in Purkinje neurons. In addition to the α subunit, the Navβ4 subunit is a key component of the Na^+ channel that contributes to the development of resurgent Na^+ current in Purkinje neurons (Grieco, Malhotra, Chen, Isom, & Raman, 2005).

In addition to Na^+ channels, K^+ channels promote action potential firing in Purkinje neurons by allowing these neurons to repolarize extremely quickly after firing (Raman & Bean, 1999). As these channels inactivate rapidly, the resulting hyperpolarization after firing is limited; this further contributes to high-frequency firing (Raman & Bean, 1999). Neurons that contain members of the Kv3 family of K^+ channels are prone to high-frequency firing (Bean, 2007), including Purkinje neurons (Akemann & Knopfel, 2006). The Kv3 class of K^+ channels also shapes burst firing in Purkinje neurons (McKay & Turner, 2004).

A third class of ion channels important for Purkinje neuron electrophysiology is that which conduct Ca^{++}. Purkinje neurons are rich in voltage-gated Ca^{++} channels, particularly in their dendritic arbors (Llinas, 1988). In slice preparations, where the dendritic tree remains intact, dendritic Ca^{++} conductances are important for bursting behaviour, characterized by a series of action potentials in quick succession, followed by an afterdepolarization that is subthreshold (McKay & Turner, 2005; Womack & Khodakhah, 2002). Burst firing constitutes one of the characteristics of the trimodal pattern of firing seen in slice preparations. The trimodal pattern consists of initial Na^+-mediated tonic firing, followed by Na^+- and Ca^{++}-driven bursts and then a period of hyperpolarized quiescence (Womack & Khodakhah, 2002; McKay & Turner, 2005). Further support for the role of dendritic conductances in trimodal firing comes from its absence in dissociated Purkinje neurons, in which the dendritic tree is removed (Fry, 2006). Dissociated Purkinje neurons, however, do fire in bursts (Raman & Bean, 1997; Swensen & Bean, 2003) and do so at membrane potentials hyperpolarized relative to that seen in tonic firing (Swensen & Bean, 2003). There are several classes of Ca^{++} channels

within Purkinje neurons, including voltage-gated P-type, which become activated at depolarized membrane potentials relative to that required for T-type channel activation (Regan, 1991). Low-threshold T-type Ca^{++} currents are the prevailing Ca^{++} current carried between action potentials during burst firing (Swensen & Bean, 2003), consistent with the tendency for this firing pattern at more hyperpolarized membrane potentials (Raman & Bean, 1997; Swensen & Bean, 2003).

In addition to direct Ca^{++} conductances via voltage-gated channels, Ca^{++} influx activates specific K^{+} channels (Ca^{++}-activated K^{+} channels); these channels are coupled to high-threshold P-type Ca^{++} channels (Womack, Chevez, & Khodakhah, 2004). These ion-activated K^{+} channels include both BK ("big") and SK ("small") channels. Such channels, named for their relative conductances, also regulate action potential firing properties (Edgerton & Reinhart, 2003; Swensen & Bean, 2003; Womack & Khodakhah, 2003). SK channels are involved in regulating spontaneous firing frequency and regularity, as blockade results in a change of firing pattern from tonic to bursting. As well, the frequency of firing within bursts significantly increases with SK channel blockade (Edgerton & Reinhart, 2003; Womack & Khodakhah, 2003). SK channels also contribute substantially to the subthreshold after-depolarization of the membrane potential that accompanies burst firing (Swensen & Bean, 2003). BK channels contribute to the after-hyperpolarization of action potentials in Purkinje neurons (Edgerton & Reinhart, 2003).

These aforementioned channels work in concert to regulate Purkinje neuron function. Perturbations to Purkinje neuron firing result from alterations in any number of these ion channels, and this can directly affect organism behaviour. Several studies

demonstrate behavioural deficits when intrinsic membrane properties are altered in Purkinje neurons. Walter, Alvina, Womack, Chevez, and Khodakhah (2006) conducted a series of experiments to examine the contributions of Purkinje neuron firing behaviour to motor abilities. In cerebellar slices, pharmacological blockade of Ca^{++} channels resulted in a decrease in firing regularity in Purkinje neurons in wild-type (WT) mice. Firing regularity was restored with addition of 1-ethyl-2-benzimidazolinone (EBIO), a SK channel activator that increases the affinity of SK channels for Ca^{++}, thereby compensating for the reduction in Ca^{++} current by channel blockade. These researchers then applied this treatment to *ducky* mice; these mice have a genetic mutation that affects P-type Ca^{++} channels, resulting in decreased Ca^{++} current density, irregular spontaneous Purkinje neuron firing, and ataxia. Perfusion of EBIO into the cerebellum of *ducky* mice significantly improved motor performance relative to vehicle-treated *ducky* control mice. Cessation of the perfusion system resulted in a return to baseline performance levels, further confirming the effectiveness of this treatment. These data provide convincing evidence of the importance of intrinsic Purkinje neuron properties to cerebellar function by demonstrating that restoration of perturbations of these properties restores cerebellar-associated motor performance.

In mice with a genetic deletion of the plasma membrane Ca^{++} ATPase subtype 2 (PMCA2), a calcium transporter protein typically present in Purkinje neurons, the frequency and regularity of spontaneous Purkinje neuron firing are decreased as a result of impaired intracellular Ca^{++} extrusion (Empson, Akemann, & Knopfel, 2010). These mice exhibit cerebellum-associated motor deficits, including impaired balance and coordination (Kozel et al., 1998). Disruption to Na^{+}-channel activity in mutant mice in

which spontaneous Purkinje neuron activity is compromised, is associated with an ataxic phenotype (Raman et al., 1997; Shakkottai et al., 2009).

Therefore, there is considerable evidence that appropriate cerebellar processing crucially depends on the integrity of "pacemaker" or spontaneous firing activity in Purkinje neurons. The intrinsic firing properties of Purkinje neurons may serve as a coding mechanism, whereby rapid and short-lived changes to the baseline firing rate of Purkinje neurons would serve as a means of encoding synaptic information received by these neurons en route to the DCN. As the exclusive output neurons of the cerebellar cortex, Purkinje neurons exert an inhibitory influence onto their target DCN neurons, which, in turn, project to other brain regions involved in the execution of motor behaviour as well as non-motor behaviours (i.e., cortical association areas) (Strick et al., 2009). This flow of information across neurons in the cerebellum is postulated to result in cerebellar inhibition of errors of movement and timing, allowing for other processing systems involved in planning, initiation, and execution of motor acts to refine their output (Albus, 1971; Raymond et al., 1996; Thach et al., 1992). Leiner, Leiner, and Dow (1989) extended this theoretical perspective to include regions of the cerebellum that project to non-motor cortical areas (i.e. the lateral cerebellum) and argued that the cerebellum contributes to mental function by refining the speed and accuracy of symbolic information processing much the way cerebellar regions interconnected with motor areas affect the accuracy and timing of motor actions.

1.7. DMD as a Cerebellar Disorder

Decades of research have confirmed the importance of the cerebellum and its principal neurons, Purkinje neurons, in the refinement of motor behaviours and precision of motor execution. The accumulation of considerable data, however, has prompted a re-evaluation and expansion of this role such that the cerebellum may be viewed as an information processor capable of coordinating and optimizing domains outside of motor function, including mental processing and cognition. It is precisely this function that is theorized to be at the core of the CNS impairments and resulting cognitive deficits associated with DMD (Cyrulnik & Hinton, 2008).

Based on the specific deficits among those with DMD (reviewed above), Hinton et al. (2004) initially proposed a characteristic cluster of cognitive impairments associated with the disorder, principally involving deficits relating to tasks requiring attention to verbal information and its repetition (deficits in recall of digits, sentence repetition, and story recall). These deficits are consistently observed in DMD patients and are preserved across levels of intellectual functioning (Hinton et al., 2000). The deficits are also thought to underlie the reported difficulty with processing phonological information, reading impairments, and academic difficulties seen more frequently in those with DMD than normally expected. Research has repeatedly demonstrated that cognitive deficits associated with DMD are not solely a consequence of decreased motor functioning. This was elucidated in studies examining age-related changes in measures of both verbal and performance intelligence and in studies comparing cognitive abilities in DMD with a similar neuromuscular disorder.

Based on cumulative research, Cyrulnik and Hinton (2008) further contended that the deficits in immediate verbal memory, or "limited verbal span", seen in those with DMD are cerebellar-mediated and arise from aberrations within the cerebrocerebellar loops emanating from the lateral cerebellum. These authors further posit that DMD may be considered a cerebellar disorder. They hypothesize that an absence of dystrophin within the cerebellum impairs the maintenance and development of phonological memory stores and information rehearsal through cerebrocerebellar loops, likely due to impaired synaptic transmission.

There are several lines of evidence that support the notion of cerebellar pathology in the absence of dystrophin, as in DMD. As previously stated, post-mortem studies have documented a decreased number of cerebellar Purkinje neurons (Jagadha & Becker, 1988; Rosman & Kakulas, 1966). Moreover, a positron emission topography (PET) study showed that glucose metabolism is reduced in DMD, in areas that typically contain dystrophin, including the cerebellum (Lee et al., 2002).

Much of the research implicating cerebellar dysfunction as a consequence of the absence of dystrophin has come from animal studies. A mouse homologue of DMD, the X chromosome-linked muscular dystrophy (*mdx*) model (Bulfield, Siller, Wight, & Moore, 1984), has been extensively used to investigate the hallmark muscle degeneration in the disorder. A spontaneous X chromosome-linked mutation results in deficiency of the full-length dystrophin isoform in both muscle and brain (Uchino, Yoshioka et al., 1994) in the *mdx* mouse. In addition, these mice show numerous musculoskeletal parallels to the human disorder, including muscle degeneration and fibrosis (Cullen & Jaros, 1988; Turgeman et al., 2008). In *mdx* mice, there is considerable muscle

regeneration (J. E. Anderson, 1998; McIntosh, Garrett, Megeney, Rudnicki, & Anderson, 1998) that leads to partial compensation and less overt clinical signs and motor consequences relative to human DMD. This characteristic of the model makes it ideal for studying cognitive deficits associated with DMD, as it lessens the difficulty in teasing apart the behavioural effects of dystrophin deficiency due to muscle-associated motor disability from those associated with an absence of brain dystrophin (Sekiguchi, 2005).

Cognitively, *mdx* mice show both learning and memory impairments, as they have difficulty in passive avoidance learning (Muntoni, Mateddu, & Serra, 1991), perform suboptimally on the T-maze, and have difficulty retaining information after long delays (Vaillend, Rendon, Misslin, & Ungerer, 1995). These dystrophic *mdx* mice, however, show no apparent deficits in procedural memory, and long-term potentiation in the hippocampus, thought to be a cellular mechanism underlying specific types of learning and memory, is preserved (Vaillend et al., 1998). As such, the cognitive deficits noted in *mdx* mice appear to be more specific than global deficits in learning and memory.

In *mdx* mice, motor function associated with the spinocerebellum, including righting and negative geotaxis, is impaired (Rafael, Nitta, Peters, & Davies, 2000). These mice also exhibit cerebellar neuronal dysfunction. Within *mdx* Purkinje neurons, the number of $GABA_A$ receptor clusters is reduced at postsynaptic sites, where dystrophin typically resides (Knuesel et al., 1999). In the absence of dystrophin, however, there are no secondary alterations in the expression of gephryin, a scaffolding protein implicated in inhibitory receptor synaptic clustering (for review, see Fritschy & Brunig, 2003), suggesting that the role of dystrophin in $GABA_A$ receptor clustering is independent of gephryin; this suggests that both proteins likely contribute to stabilize receptor clusters

via distinct mechanisms and pathways. Kueh, Head, and Morley (2008) found no difference in the expression of the $GABA_A$ receptor α-1 subunit protein within the cerebellum between *mdx* and control mice. This observation suggests that dystrophin may not regulate receptor protein expression *per se*, but rather plays a role in clustering and maintenance of receptors at postsynaptic sites. Consistent with this interpretation is the finding that the number of extrasynaptic $GABA_A$ receptor subunits is increased in *mdx* Purkinje neurons (Kueh, Dempster, Head, & Morley, 2011).

Research has also demonstrated that synaptic transmission is impaired in the absence of dystrophin. There is a reduction in the frequency and amplitude of miniature inhibitory postsynaptic currents (mIPSCs) in *mdx* compared to wild-type Purkinje neurons, consistent with fewer $GABA_A$ receptor sites upon which the neurotransmitter can induce its synaptic effects (J. L. Anderson, Head, & Morley, 2003; Kueh et al., 2008). The addition of bicuculline abolished the vast majority of mIPSCs, confirming they were GABA-ergic (J. L. Anderson et al., 2003). Alterations in mIPSCs may occur as a result of pre- or post-synaptic cellular changes, either due to increased probability of neurotransmitter release at the presynaptic terminal or due to changes in postsynaptic receptor expression. In support of the idea that postsynaptic alterations decrease inhibitory input, the level of paired-pulse facilitation, which is presynaptically mediated and associated with an increased probability of neurotransmitter release, is normal in *mdx* Purkinje neurons (J. L. Anderson, Head, & Morley, 2004).

In another experiment demonstrating alterations in synaptic transmission in *mdx* mouse cerebellum (J. L. Anderson et al., 2003), baseline evoked excitatory postsynaptic potentials (EPSPs) were recorded from Purkinje neurons after stimulation of the

molecular layer to gather baseline values. EPSPs were then recorded in the presence of bicuculline, which abolishes $GABA_A$-mediated inhibitory input. A net increase in the amplitude of ESPSs was found in both WT and *mdx* mice, however the magnitude of this increase was diminished in the *mdx* mice relative to controls, demonstrating a reduction in the inhibitory drive to Purkinje neurons.

Further evidence for the role of dystrophin in synaptic function comes from investigations into synaptic plasticity within cerebellar Purkinje neurons in dystrophic mice. Within the cerebellum, depression of the postsynaptic potential strength at parallel fibre-Purkinje neuron synapses is induced following concomitant activation by CFs and parallel fibres; these are the two sources of excitatory input to the cerebellum (Ito & Kano, 1982). This depression, termed LTD, is thought to underlie cerebellar-mediated learning. In *mdx* mice, the capacity for LTD is suppressed (J. L. Anderson et al., 2004). In this study, LTD was induced in the presence of a $GABA_A$ receptor blocker, and findings therefore suggest that dystrophin deficiency also impairs GABA-independent synaptic transmission.

In another form of LTD, homosynaptic LTD, the synaptic strength of excitatory postsynaptic potentials at the parallel fibre-Purkinje neuron synapse is reduced with repeated parallel fibre stimulation alone, in the absence of concomitant CF stimulation. Homosynaptic LTD has been examined in *mdx* mice (J. L. Anderson, Morley, & Head, 2010). Here, Purkinje cells from *mdx* mice displayed an increased magnitude of homosynaptic LTD relative to wild-type controls. Although this was in contrast to their previous report of decreased classical heterosynaptic LTD (J. L. Anderson et al., 2004), the latter study by these authors demonstrated that cellular mechanisms of synaptic

plasticity and learning in the cerebellum appeared to be altered in the absence of dystrophin. However, the role of homosynaptic LTD in cerebellar learning and functioning is poorly understood.

1.8. Thesis Rationale

Given the characteristically high expression of dystrophin in cerebellar Purkinje neurons, it stands to reason that the absence of this protein will perturb neuronal function. A considerable body of work suggests that dystrophin deficiency may also underlie the cognitive deficits seen in DMD. However, the precise role of dystrophin in the CNS, in particular in the cerebellum, has not been identified, nor is there a clear understanding of the mechanisms by which a lack of dystrophin at the cellular level impairs cognitive function.

There are several lines of converging evidence that point to cerebellar dysfunction as central to the noted cognitive deficits in DMD. Firstly, at a cellular level, dystrophin expression is highest in this brain region where it resides exclusively in Purkinje neurons, the key input-output regulator of this structure. Secondly, the subcellular localization of dystrophin to postsynaptic neuronal sites, its extensive colocalization with receptors at inhibitory synapses, and its colocalization with DGC constituents that interact with ion channels integral to neuronal function all strongly implicate this protein in accurate Purkinje neuron function. Consequently, these features also suggest corresponding deficits in the absence of dystrophin. Thirdly, studies of those with DMD demonstrated cerebellar aberrations, including Purkinje neuron loss (Jagadha & Becker, 1988; Rosman & Kakulas, 1966) and impairments in glucose utilization (Lee et al., 2002). Fourthly,

specific cognitive dysfunctions reported in DMD, including those in immediate verbal memory and phonological processing, are associated with disrupted cerebrocerebellar functioning (Cyrulnik & Hinton, 2008). Lastly, research using the *mdx* model of DMD has elucidated cerebellar deficits at the neuronal level, including impaired clustering of neurotransmitter receptors (i.e. $GABA_A$) (Knuesel et al., 1999), impaired synaptic signalling (J. L. Anderson et al., 2003), and altered synaptic plasticity (J. L. Anderson et al., 2004; 2010).

Despite the abundance of evidence implicating the cerebellum in DMD, no study to date has thoroughly examined dystrophin localization within the lateral cerebellum, the region associated with non-motor, cognitive aspects of function, in relation to the vermal region. Moreover, the intrinsic electrophysiological properties of dystrophin-deficient Purkinje neurons have not been elucidated. Examining intrinsic electrophysiological properties of Purkinje neurons is crucial for understanding cerebellar functioning in DMD and is essential for determining whether the inherent information processing ability of the cerebellum is compromised at the cellular level in DMD.

The current research examined possible cerebellar pathophysiology associated with DMD using the *mdx* model. In particular, this thesis work focused on the lateral cerebellum that is implicated in cognitive function. The research investigated the regional and subcellular localization of dystrophin in both vermal and lateral cerebellum in wild-type mice. In the second part of the study, the electrophysiological phenotype of Purkinje cells from wild-type and dystrophic cerebellum, both vermal and lateral regions, was characterized. Specifically, the following hypotheses were tested:

1) dystrophin is localized to cerebellar Purkinje cells, specifically in dendritic and somatic membranes in both vermal and lateral cerebellum. However, this distribution will differ quantitatively between regions.

2)Purkinje neurons from the cerebellum of mdx mice, including the lateral region, will exhibit altered electrophysiological properties, including altered spontaneous firing rates and action potential properties relative to those from wild-type cerebellum in the same region.

The experiments reported in the next two chapters were designed to test these hypotheses. Results of these experiments are detailed in the two chapters. Chapter 2 is a manuscript (unpublished as yet) in which I did the work and drafted the manuscript, with input from Dr. Judy Anderson and Dr. Mark Fry. Chapter 3 is in manuscript form (not yet submitted) in which I did the work and drafted the manuscript, with input from Dr. Fry. The final chapter of the thesis, Chapter 4, integrates the findings of these two major experiments toward a synthesis with the literature, and identifies possibilities for future investigation.

Chapter 2

Experiment 1: Distribution of Dystrophin Protein in the Lateral and Vermal Mouse Cerebellum

Objective: To characterize regional (vermal and lateral) and subcellular dystrophin localization in Purkinje neurons in normal control mouse brain.

This experiment characterized dystrophin distribution within the cerebellum of WT mice using a highly specific dystrophin antibody, a necessary step in understanding the effects of its absence in DMD. This research is the first to examine in detail the presence of dystrophin in a cerebellar region implicated in cognitive function and hypothesized to account for cognitive deficits reported in DMD.

Abstract

Dystrophin, present in muscle, also resides in the brain where it is preferentially localized to cerebellar Purkinje neurons. The cerebellum, although historically associated with motor abilities, is also implicated in cognition. An absence of brain dystrophin in dystrophin-deficient *mdx* mice results in impairments in learning and memory. Localization studies of cerebellar dystrophin, however, have focused on the vermal cerebellum, the region associated with motor function, and have not investigated dystrophin distribution in the lateral cerebellum, considered to mediate cognitive functions. The present study examined dystrophin localization in vermal and lateral cerebellar regions and across subcellular areas of Purkinje neurons in wild-type (WT) mice using immunohistochemistry. In both vermal and lateral cerebellum, dystrophin was restricted to puncta on somatic and dendritic membranes of Purkinje neurons. The density of dystrophin puncta was greater in the lateral than the vermal region. Neither the size of puncta nor the area of Purkinje neuron somata differed between regions. Results support the view that cognitive deficits in the *mdx* model may be mediated by the loss of dystrophin, particularly in the lateral cerebellum. Findings have important implications for future studies examining the neurophysiological sequelae of neuronal dystrophin deficiency and the role of the lateral cerebellum in cognition.

2.1. Introduction

In Duchenne muscular dystrophy (DMD), absence of the protein dystrophin leads to fatal muscle weakness (Wallace & McNally, 2009). Cognitive deficits are also reported among those with DMD, including mean IQ scores one standard deviation below the population mean and a higher incidence of mental retardation (Cotton et al., 2001). Impaired performance on the Digit Span test, a measure of immediate verbal memory, is a common finding among those with DMD (S. W. Anderson et al., 1988; Dorman et al., 1988; Leibowitz & Dubowitz, 1981; Ogasawara, 1989; Whelan, 1987). In addition to muscle, the dystrophin gene is also expressed in the nervous system, yielding various-sized dystrophin isoforms, including truncated dystrophin isoforms (Dp71, Dp116, Dp140, and Dp260) (as reviewed in Perronnet & Vaillend, 2010). Full-length dystrophin protein, Dp427, resides in specific populations of neurons involved in learning and memory, including cerebellar Purkinje neurons and pyramidal neurons of the hippocampus and cerebral cortex in the normal brain (Huard & Tremblay, 1992; Lidov et al., 1990; Uchino, Teramoto, Naoe, Yoshioka et al., 1994) but is absent in DMD brains (T. W. Kim et al., 1995; Uchino, Teramoto, Naoe, Yoshioka et al., 1994). Localization of full-length neuronal dystrophin in the normal brain, however, is heterogeneous. For example, strong dystrophin immunoreactivity was found in nearly all pyramidal neurons in the deep layers of the frontal cortex, whereas many parietal Layer II and III pyramidal neurons lacked dystrophin (Lidov et al., 1990). In the hippocampus, only a subset of dystrophin-positive hippocampal dendritic spines was detected, whereas all spines in the cerebellar molecular layer and Layers II and III of the cerebral cortex displayed dystrophin (Jancsik & Hajos, 1998).

Dystrophin plays a key role in maintaining acetylcholine receptors in muscle (Grady et al., 2000; Kong & Anderson, 1999) and is implicated in synaptic receptor clustering in neurons (Lidov et al., 1990; 1993). Dystrophin colocalizes with a subset of $GABA_A$ receptor clusters in neurons of the cerebral cortex, hippocampus, and cerebellum (Knuesel et al., 1999). Several insights into the consequences of an absence of brain dystrophin have been garnered using the *mdx* mouse model of DMD, in which neuronal and muscle dystrophin are absent (Uchino, Yoshioka et al., 1994), including a reduction in postsynaptic $GABA_A$ receptor clusters in cerebellar Purkinje neurons (Knuesel et al., 1999; Kueh et al., 2011) and an increase in extrasynaptic $GABA_A$ receptor clustering (Kueh et al., 2011), further implicating dystrophin in the maintenance of GABAergic receptors. At the synaptic level, inhibitory input to Purkinje neurons is reduced in *mdx* mice (J. L. Anderson et al., 2003), as are the amplitude and frequency of miniature inhibitory post-synaptic currents (Kueh et al., 2008), attributed to the reduction in $GABA_A$ receptor clusters. Synaptic plasticity is compromised in the absence of dystrophin, as long-term depression of the parallel fibre-Purkinje neuron synapse is aberrant in *mdx* mice (J. L. Anderson et al., 2004; J. L. Anderson et al., 2010). These mice display impairments in learning and memory, including difficulty in passive-avoidance learning (Muntoni et al., 1991) and retaining information after long delays, as assessed with a bar-pressing task (Vaillend et al., 1995). As well, memory performance on the T-maze is suboptimal in *mdx* mice (Vaillend et al., 1995).

Although research has confirmed molecular and neurophysiological dysregulation in dystrophin-deficient cerebellum, research investigating the cerebellum's contribution to the noted cognitive impairments in the absence of neuronal dystrophin is

hampered by the fact that all studies thus far have focused on the vermal region, where damage leads to impaired motor behaviour in both mice and humans, affecting coordination and equilibrium (Joyal et al., 1996; 2001; Nyberg-Hansen & Horn, 1972). In contrast, there is no detailed investigation of dystrophin in the lateral cerebellum. Studies documenting lateral cerebellar activation during non-motor mental activity in humans, including the generation of verbs when presented with nouns (Petersen et al., 1989), silent counting (Decety et al., 1990), mental imagery (Ryding et al., 1993), and completion of a pegboard puzzle (S. G. Kim et al., 1994), have implicated this cerebellar region in cognition.

Immunohistochemical studies using antibodies that detect all brain isoforms have investigated dystrophin localization in cerebellar coronal sections and report heterogeneous labeling of Purkinje neurons (Blake et al., 1999; Moukhles & Carbonetto, 2001). Details on precise labeling across functional zones of the cerebellum, however, are not provided. Moreover, findings regarding subcellular neuronal dystrophin localization have been inconsistent. Studies reported abundant cytoplasmic staining of full-length dystrophin in mouse cerebellum (Huard & Tremblay, 1992; Huard, Satoh, & Tremblay, 1992), contradictory to previous reports where cytoplasmic labeling was negligible (Knuesel et al., 1999; Lidov et al., 1990). Given the contradictory findings on full-length dystrophin localization in Purkinje neurons and its heterogeneous pattern in other brain regions, this study aimed to: 1) conduct a detailed examination of dystrophin localization in Purkinje neurons of the lateral cerebellum, the region associated with cognitive processing, using an antibody specific to full-length neuronal dystrophin (herein referred to as “dystrophin”); 2) determine whether its subcellular distribution is consistent with

that reported for the vermal cerebellum; and, 3) quantitatively compare dystrophin localization between these two regions in WT mice.

2.2. Materials and Methods

2.2.1. Animals and Tissue Processing

Six-week old C57BL6 mice (n=6) housed at the University of Manitoba were sacrificed by inhalation of isoflurane, followed by cervical dislocation, as approved by the institutional Animal Care and Use Protocol Review Committee. The cerebellum was removed, placed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) (pH 7.4), and infiltrated overnight, followed by overnight immersion in cryoprotectant (30% sucrose + 0.1% sodium azide in PB). Vermal and lateral areas were isolated and placed cut-side down in cryomolds containing Cryomatrix (Shandon; Thermo Scientific, Canada). Molds were frozen in isopentane at –55 ºC and stored at –20 ºC. Sagittal vermal and parasagittal lateral cryosections were cut to a thickness of 8 µm, mounted onto gelatin-coated slides, air-dried, and stored at –20 ºC.

2.2.2. Double Immunofluorescent Labelling

Sections were brought to room temperature (RT) and washed in PB. As immunohistochemical detection of dystrophin is sensitive to aldehyde fixation (Lidov et al., 1993), antigen retrieval with microwave irradiation of sections in 0.01M citrate buffer (pH 6.0) for 3.5 minutes was used to expose epitopes. Sections were cooled to RT in citrate buffer, washed in PB, and blocked for 2 hours at RT in 10% normal goat serum containing 0.3% Triton-X, 10% glycine and Fab fragment of unconjugated goat anti-

mouse IgG antibody (diluted 1:10) in PB. Sections were incubated overnight at 4 ºC in vehicle (10% normal goat serum + 0.03% Triton-X in PB) containing Dys1 (NovoCastra; UK), a mouse monoclonal antibody raised against the rod domain of full-length dystrophin (diluted 1:10), and anti-calbindin (Santa Cruz; US), a rabbit polyclonal antibody raised against the calcium-binding protein calbindin D28k, a Purkinje-neuron marker (diluted 1:100). Following washes in PB, sections were incubated in vehicle simultaneously with goat anti-mouse DyLight 649 (Jackson Immunoresearch; US) (diluted 1:200) and goat anti-rabbit Alexa 555 (Invitrogen; Canada) (diluted 1:1000) for 1 hour at RT for fluorescent detection of dystrophin and calbindin respectively. After washing with PB, sections were coverslipped in ProLong mounting medium (Invitrogen; Canada). Slides were sealed with nail polish and stored at 4 ºC. Sections of mouse quadriceps muscle were processed using the same protocol to confirm dystrophin immunoreactivity (positive control). Cerebellar sections processed without Dys1 during the primary antibody incubation served as negative controls.

2.2.3. Image Acquisition

Fluorescent immunostaining was detected using a Zeiss Axio Imager.Z1 (Carl Zeiss Canada Ltd.) and images captured with Axiocam (Carl Zeiss Canada Ltd.). Pseudocolor (green) was applied to the Alexa 555 fluorescence (anti-calbindin) after image acquisition. Using the 40x objective (EC Plan-Neofluar, 1.3 NA, oil) and Zeiss Apo Tome (Carl Zeiss Canada Ltd.), the Purkinje neuron and molecular layers were visualized in one field of view (FOV) for dystrophin quantification (resolution 265 nm/pixel). Exposure times were kept constant for both dystrophin and calbindin

immunoreactivity during image acquisition for quantification. Only areas in which primary dendrites were attached to the Purkinje somata were chosen for quantification. Images were captured with 20x (Plan-Apochromat, 0.8 NA) and 100x (Plan-Apochromat, 1.4 NA, oil) objectives to examine cellular and subcellular localization of dystrophin. Z-stacked images were taken with a 100x objective to give a representative view of dystrophin across the entire z-axis of the section. Care was taken to ensure the cerebellum was sectioned such that the dendritic arbor was in the proper plane of orientation. In the vermal region, the plane of the dendritic tree of most Purkinje neurons is parallel to the plane of the slice. As one moves laterally, however, this plane of sectioning must be slanted to maintain a parallel plane with the dendritic tree. Although we were successful at obtaining FOVs from lateral cerebellum with this desired plane, it was not possible to ensure this plane was consistent for any given lobule, thereby precluding us from conducting lobule-specific analyses of dystrophin puncta.

2.2.4. Quantification of Immunofluorescence

From each FOV, measurements were taken from the soma, proximal dendritic and distal dendritic areas of Purkinje neurons. Proximal dendrites were defined as single dendrites arising directly from the soma to a distance of 50 µm. Distal dendrites were defined as extending from the end of the proximal dendrites distally through the molecular layer. Areas 50 µm x 50 µm were cropped from each FOV for dystrophin quantification in proximal and distal dendrites. Purkinje neuron somata were traced manually. Digital images were exported as TIFF files, and data were collected using the "Particle Analysis" function of ImageJ software (http://rsbweb.nih.gov/ij/). As the values

for dystrophin puncta number and size varied based on the threshold of illumination applied in ImageJ, the number of dystrophin-positive puncta were counted manually from single-channel images of dystrophin fluorescence in a sample from each region (vermal and lateral) from each subcellular location (soma, proximal dendrites, distal dendrites). The illumination threshold that detected the number of puncta most similar to values obtained manually was consistently used between groups and across subcellular locations to ensure reliability during data collection. To ensure that blood vessels were excluded from analysis, preliminary counts were conducted on images in which blood vessels were clearly visible. The percentage of puncta under 2 μm^2 in these images was 98%. Based on this estimate and the range of blood-vessel diameters, the criterion for inclusion in the analysis was established as puncta ranging from a minimum of 0.1 μm^2 (Knuesel, Zuellig, Schaub, & Fritschy, 2001) to a maximum of 2 μm^2, resulting in exclusion of the upper 2% of dystrophin-positive puncta from analysis, which were predicted to represent labelling of blood vessels. Images were coded to prevent specific knowledge of cerebellar regions during data collection.

2.2.5. Statistical Analysis

Data were collected from a total of 96 images (4 FOVs x 4 sections x 6 animals) per cerebellar region. Values from four FOVs for each subcellular location per section were averaged for analysis. Puncta number was standardized by dividing the number of puncta by the area analyzed (density). A 2 x 3 x 4 (Region x Subcellular x Section) nested repeated-measures analysis of variance (ANOVA) was conducted to test for differences in the average size of dystrophin puncta and puncta density between regions

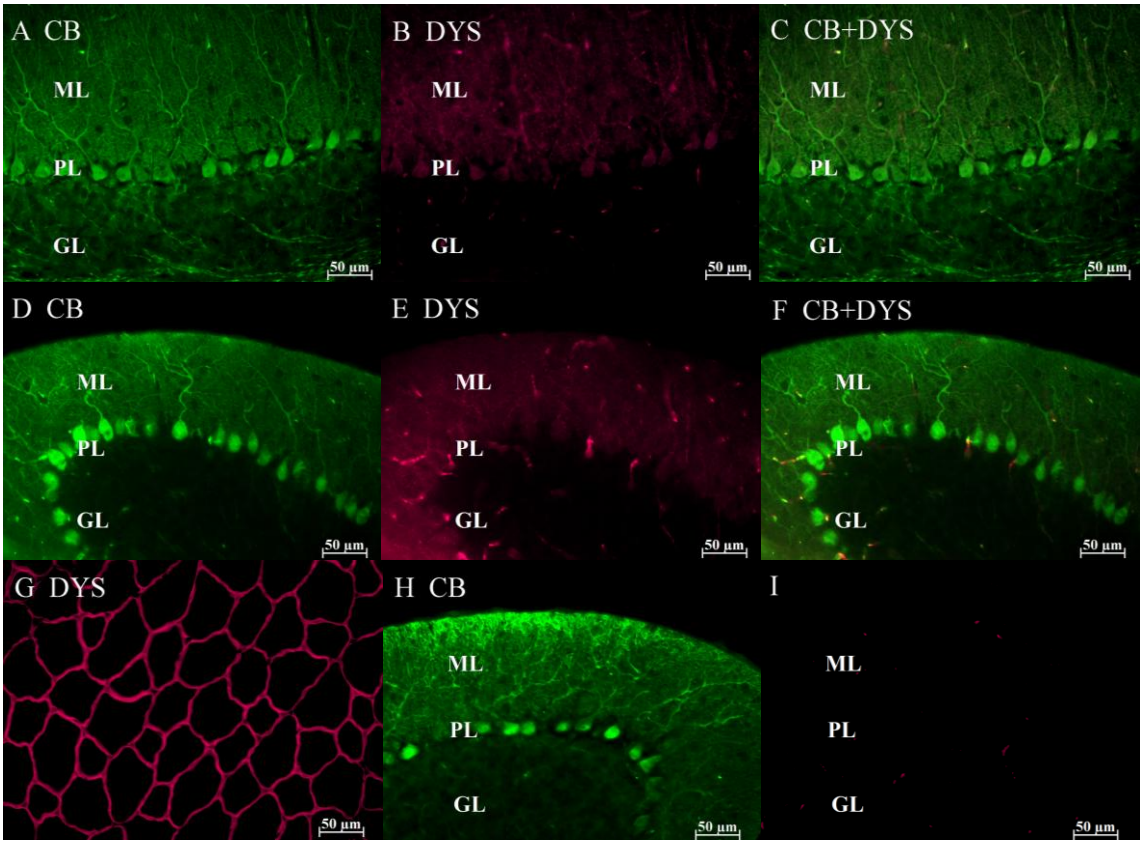
and across subcellular locations. A 2 x 4 (Region x Section) repeated-measures ANOVA was used to test for regional differences in soma size. Fisher's *post hoc* tests were performed to determine which of the multiple comparisons showed statistical significance (set at $p < 0.05$). All tests were two-tailed, and analyses were performed using Statview version 5.0.1 for Macintosh (SAS Institute Inc., 1998). All data are expressed as mean ± standard error of the mean (SEM).

2.3. Results

2.3.1 Qualitative Analysis of Dystrophin Localization

Within the vermal cerebellum of WT mice, dystrophin was localized as discrete puncta restricted to the Purkinje neuron layer and molecular layer of the cerebellar cortex (Figure 2.1A-C) and was identified as representing dystrophin along Purkinje somatic and dendritic membranes, which were delineated by calbindin labelling. Dystrophin was absent in the granule layer containing axons of Purkinje neurons. Dystrophin was detected in the lateral cerebellum (Figure 2.1D-F) in a pattern consistent with that seen in the vermal region, with immunoreactivity present in all Purkinje neurons examined. Positive-control sections of quadriceps muscle showed bright uniform immunofluorescence at the fibre periphery (Figure 2.1G), as expected (Huard & Tremblay, 1992). Negative-control sections from vermal cerebellum did not show immunoreactivity within Purkinje neuron somata or dendrites (Figure 2.1H), although some reactivity persisted within blood vessels, likely due to non-specific binding from anti-mouse secondary antibodies (Figure 2.1I).

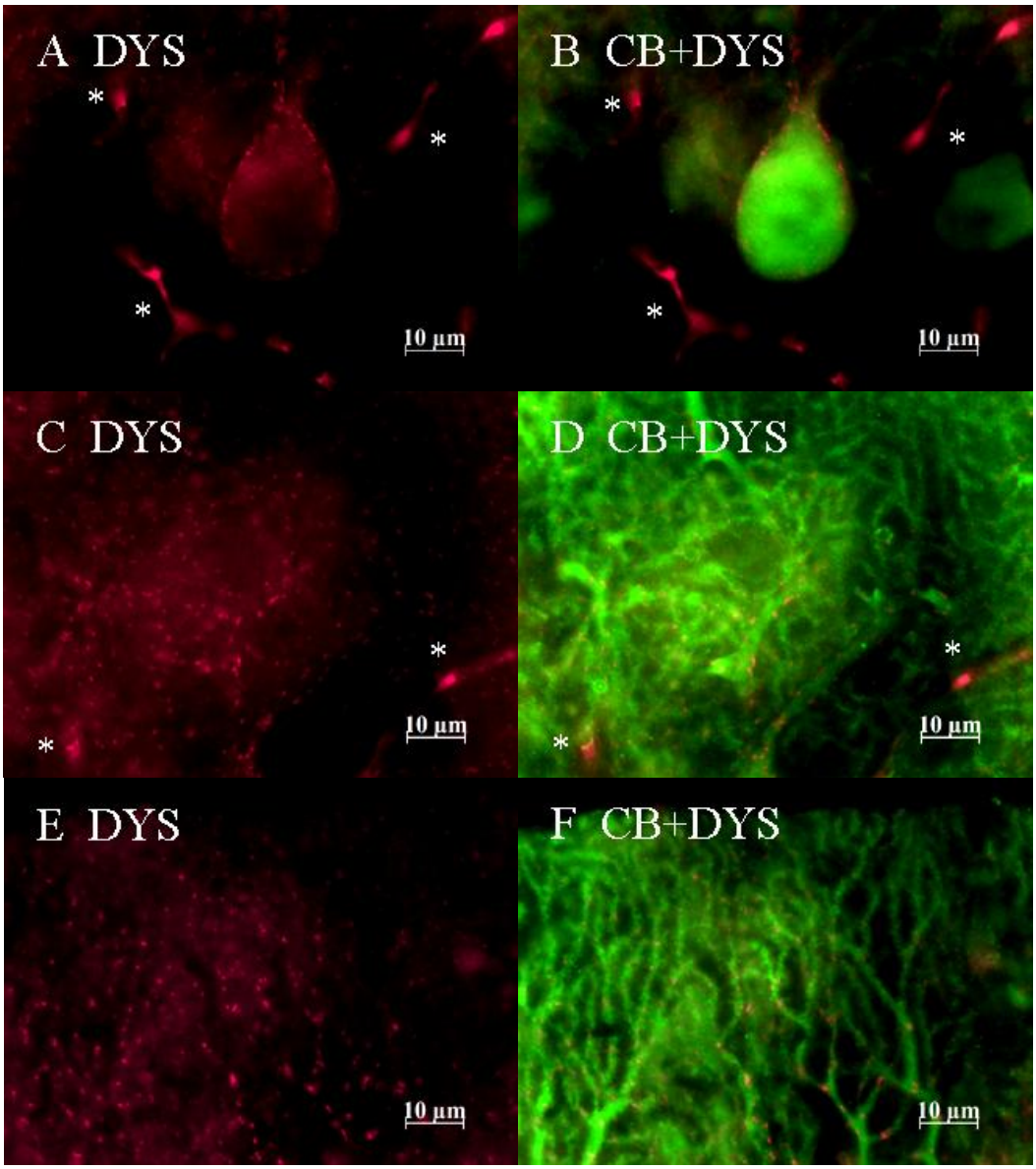
Figure 2.1. Double immunofluorescently-labelled cryosections (8 µm) of vermal (A-C) and lateral (D-F) cerebellum stained for calbindin (CB) (green) and dystrophin (DYS) (red) at 20x magnification. In both regions, neuronal dystrophin is confined to the molecular (ML) and Purkinje neuron layer (PL) but is devoid in Purkinje neuron axons and other neurons in the granule layer (GL). Intensely bright dystrophin staining represents immunoreactivity within vasculature. (G) Cryosection (5 µm) of mouse quadriceps muscle, with intense dystrophin labelling around the fibre periphery (positive control). (H) & (I) show single-channel images of cryosection (8 µm) of vermal cerebellum incubated with the standard immunofluorescent reagents, including calbindin (CB) (green), with the exclusion of the dystrophin antibody (negative control).



At 100x magnification, dystrophin puncta were clearly visible along the somatic membrane surrounding the calbindin-positive Purkinje neuron cytoplasm (Figure 2.2A-B). Dystrophin puncta along the proximal dendritic membrane completely aligned with calbindin labelling in both regions (Figure 2.2C-D) and extended through the entire molecular layer to the most distal dendrites (Figure 2.2E-F). As the distribution pattern of

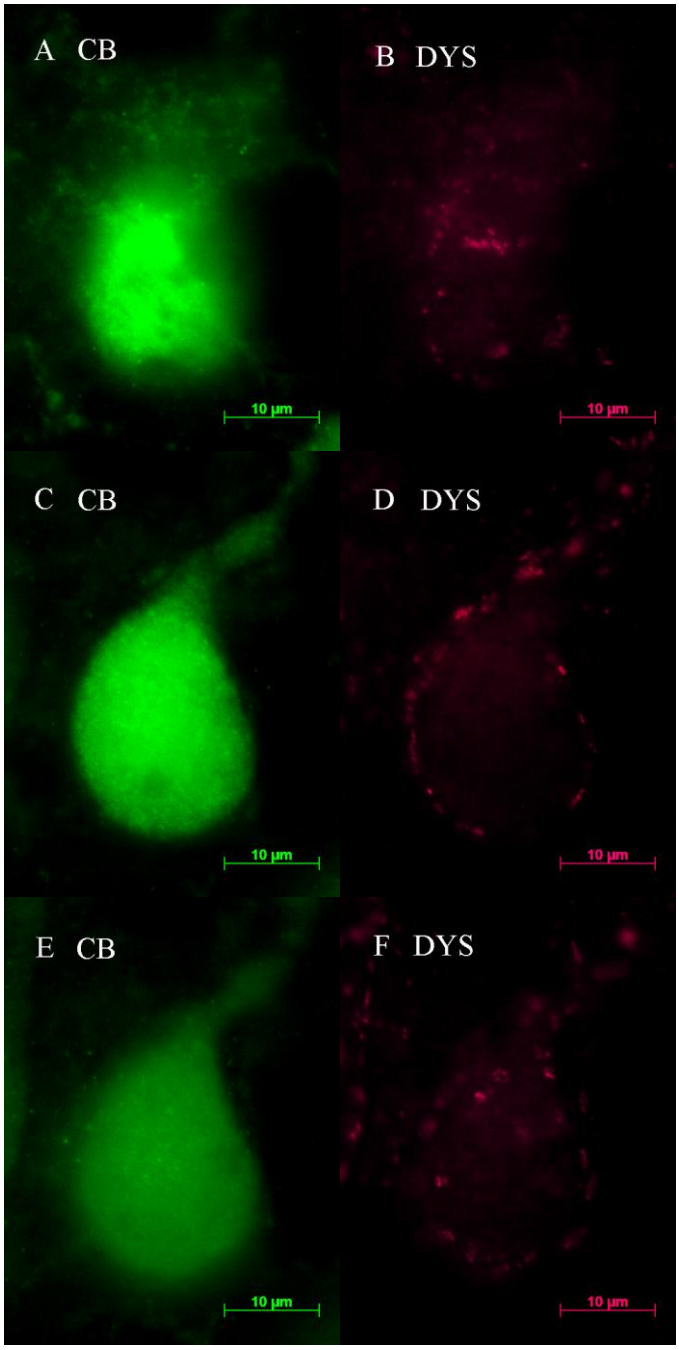
dystrophin was consistent between cerebellar regions, figures show representative images from the lateral cerebellum.

Figure 2.2. Single-channel (A, C, and E) and dual-channel (B, D, and F) images (100x magnification) of Purkinje neuron soma (A, B), proximal (C, D), and distal dendrites (E, F) in cryosections (8 µm) of lateral cerebellum stained for dystrophin (DYS) (red) and calbindin (CB) (green). Dystrophin is restricted to somatic membranes surrounding the cytoplasmic calbindin in a punctate fashion (A, B). Dystrophin resides along the entire Purkinje neuron dendrite, including proximal dendrites immediately extending from the soma (C, D), and distal dendrites at the apex of the ML (E, F). *denotes blood vessels.



The possibility of the presence of cytoplasmic dystrophin was investigated by examining z-stacked images. Figure 2.3 shows that these apparently cytoplasmic dystrophin puncta were in focus when the calbindin-positive soma was at the top of the focal plane. As the focal plane changed, the puncta became more diffuse and fainter, while other puncta surrounding the soma became brighter and more focal. We interpret these observations to indicate that puncta are restricted to the somatic membrane.

Figure 2.3. Purkinje neuron soma (100x) in a cerebellar vermal cryosection (8 µm) immunofluorescent for calbindin (CB) (green; A, C, E) and dystrophin (DSY) (red; B, D, E) through focal planes. (A) At the top of the section, when the Purkinje neuron soma is not in focus, as labeled by CB, dystrophin puncta appear bright and in focus in the centre of the soma (B). (C) As the focal plane changes, revealing the cross-sectional view of the Purkinje neuron, labeled by CB, bright dystrophin labeling is confined to the somatic membrane (D). (E) As the focal plane continues along the z-axis, and the CB-positive Purkinje cell soma becomes out of focus, more bright puncta appear in the centre of the cell (F), confirming the presence of dystrophin around the neuronal membrane.



2.3.2. Quantitative Analysis of Dystrophin

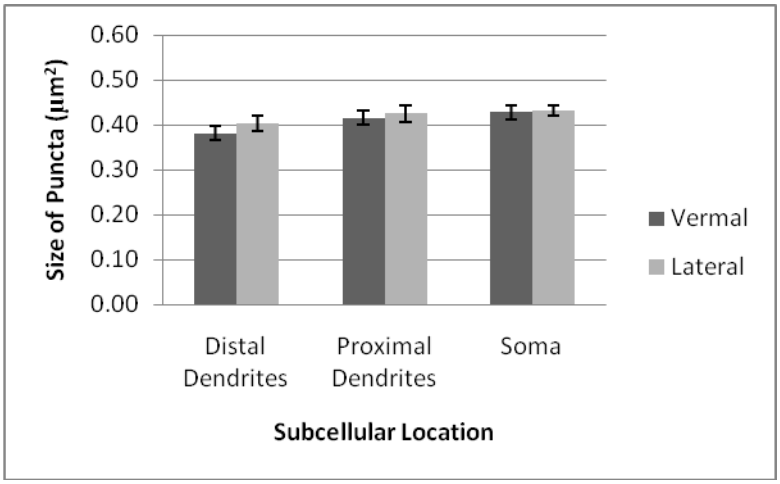
2.3.3. Soma Size in Vermal and Lateral Cerebellum

Soma size was measured to examine possible morphological differences in Purkinje neurons as a function of region. There was no significant difference in soma area between cerebellar regions ($F_{(1,10)} = 1.01$, $p = 0.34$; mean soma size vermal = 395.75 ± 7.5 μm^2; mean soma size lateral = 380.36 ± 9.1 μm^2).

2.3.4. Size of Dystrophin Puncta

The mean area of dystrophin puncta did not differ between vermal and lateral cerebellum ($F_{(1,30)} = 0.78$, $p = 0.38$; vermal = 0.408 ± 0.01 μm^2; lateral = 0.423 ± 0.009 μm^2). The mean size of puncta was similar across subcellular locations ($F_{(2,30)} = 2.5$, $p = 0.09$; distal dendrites = 0.393 ± 0.012 μm^2; proximal dendrites = 0.420 ± 0.012 μm^2; soma = 0.430 ± 0.01 μm^2). There was no interaction between region and subcellular location for punctum size ($F_{(2,30)} = 0.15$, $p = 0.86$). Therefore, the size of dystrophin puncta was similar between regions and across subcellular locations (Figure 2.4).

Figure 2.4. Mean size (± SEM) of dystrophin-positive puncta in Purkinje neurons as a function of cerebellar regions and subcellular location (n = 24).

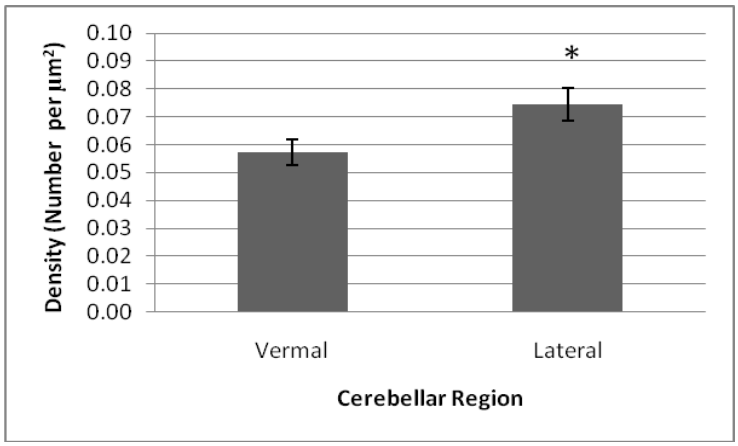


2.3.5. Density of Dystrophin Puncta

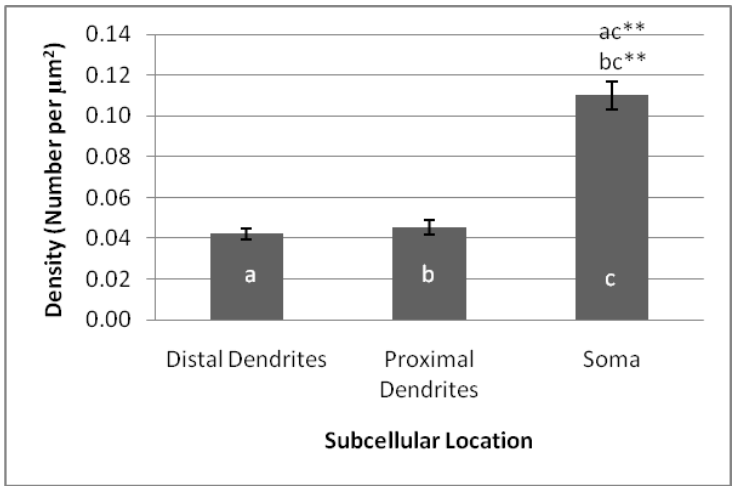
The density of dystrophin puncta was significantly higher in the lateral cerebellum relative to the vermal region ($F_{(1,30)}$ = 6.89, p = 0.01; vermal region= 0.057 ± 0.005/ µm^2; lateral region = 0. 074 ± 0.006/ µm^2) (Figure 2.5a). The density of puncta also varied by subcellular location ($F_{(2,30)}$ = 45.71, p = <0.0001). Puncta density was two-fold higher in the soma (0.11 ± 0.007/ µm^2) than in either the proximal dendritic (0.045 ± 0.004/ µm^2; p < 0.0001) or distal dendritic areas (0.042 ± 0.003/ µm^2; p < 0.0001), as determined by Fisher's *post hoc* tests. The density of dystrophin puncta did not differ between proximal or distal dendritic regions (p = 0.63) (Figure 2.5b). There was no Region x Subcellular location interaction ($F_{(2,30)}$ = 0.66, p = 0.52), indicating overall that the number of dystrophin puncta, irrespective of subcellular location, was greater in the lateral than the vermal region (Figure 2.5c).

Figure 2.5. Mean density of dystrophin-positive puncta (± SEM) in Purkinje neurons between cerebellar regions and across subcellular locations. (A) The density of dystrophin puncta was significantly greater in lateral vs. vermal cerebellum (*p = 0.01; n = 72) and (B) was significantly higher in the soma than either the proximal (**p < 0.0001) or distal dendritic regions (**p < 0.0001; n = 48). (C) Mean density of dystrophin puncta as a function of cerebellar region by subcellular location.

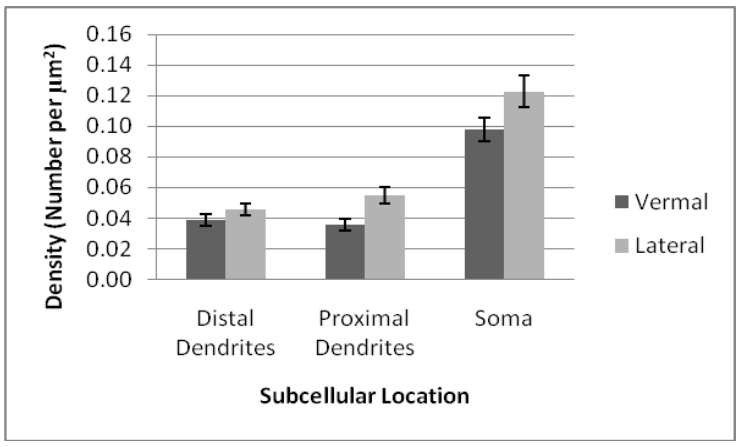
A



B



C



2.4. Discussion

The present study of mouse cerebellum extends our understanding of the specific distribution of dystrophin in the mammalian brain. This is the first detailed report of dystrophin localization along Purkinje neuron somata and dendritic membranes in lateral cerebellum, as was previously reported for the vermal cerebellum (Huard & Tremblay, 1992; Knuesel et al., 2000; Lidov et al., 1990; Lidov et al., 1993). This observation is important, given the recent implication of the lateral cerebellum in non-motor aspects of function, including cognition, and the published reports of heterogeneous dystrophin localization in other CNS regions (Lidov et al., 1990; Jancsik & Hajos, 1998). The lack of cytoplasmic labelling of dystrophin, as imaged through the focal plane of the Purkinje soma, further corroborate the postsynaptic localization of dystrophin and extend this to consideration of cerebellar functional regions.

The present finding of increased dystrophin density within the lateral regions has important implications for future research examining the putative role of the cerebellum in cognitive dysfunction in the dystrophin-deficient brain. Insights into the relative contribution of the cerebellum to the documented learning and memory deficits in the *mdx* model have examined only the vermal cerebellum, the region involved in motor behaviour and balance. This fact is concerning, given that the *mdx* model has been used extensively as a means of investigating dystrophin deficiency, as is the case in DMD. Generalizing cerebellar neurophysiological findings from the *mdx* mouse to cognitive impairments in DMD is not without limitations, which are exacerbated by the lack of focus on the more relevant cerebellar functional zone in mouse studies. In light of the

present findings, future research investigating cerebellar involvement, as it pertains to cognition, in the *mdx* model should focus on the lateral region.

One intriguing explanation for the 30% higher density of dystrophin in the lateral cerebellum is that there are simply more $GABA_A$ receptor clusters in this region, since dystrophin colocalizes with $GABA_A$ receptor subunits. In the present study, the density of dystrophin puncta within distal dendrites from the vermal region (0.04 puncta/ μm^2) was similar to the density reported by others (Knuesel et al., 1999) for the constituent $GABA_A$ subunit found in mature Purkinje neurons, the α1 subunit, in the molecular layer in similarly aged mice (0.0367/ μm^2). However, even if $GABA_A$ receptor clusters vary in number between functional zones of the cerebellum, this may not fully account for the greater density of dystrophin puncta in the lateral region, since the stoichiometry of the colocalization is not one-to-one (Knuesel et al., 1999). It will be important to determine if $GABA_A$ receptor localization and/or activity differs between cerebellar functional regions.

The physiological effects of dystrophin loss from Purkinje neurons on the function of the lateral cerebellum are not known. Given that local neuronal circuitry is preserved across the entire cerebellum (Ito, 2006), synaptic deficits similar to those reported in the vermis may occur in the lateral region in the *mdx* model with more prominence, given the greater distribution of dystrophin in the lateral cerebellum reported. As previously stated, *mdx* mice are impaired in both learning and memory tasks (Muntoni et al., 1991; Vaillend et al., 1995). As well, hippocampal-dependent memory retention of spatial learning, as shown by performance in the Morris water maze, is diminished in these mice (Vaillend, Billard, & Laroche, 2004). The lateral cerebellum is

also implicated in spatial learning, as rats with lateral cerebellar lesions performed suboptimally on the Morris water maze with no concomitant impairment in motor performance or coordination (Joyal et al., 1996; 2001) By comparison, rats with vermal lesions show impaired visual guidance to the target platform (Joyal et al., 1996). These reports illustrate the discrete functional differences between the vermal and lateral cerebellum. Deficits in spatial learning in the *mdx* mouse suggest the possibility that signaling in the lateral cerebellar hemisphere is dysfunctional due to an absence of dystrophin. The ability to dissociate cerebellar involvement in a task that is highly dependent upon intact hippocampal functioning, however, is problematic, particularly as both hippocampal and cerebellar neurons lack dystrophin in the *mdx* mouse. Tests of function in the *mdx* mouse using cerebellar-associated learning paradigms, such as the conditioned eyeblink response (as reviewed in Villarreal & Steinmetz, 2005) could determine the extent of synaptic perturbation in the cerebellum as a result of the genetic loss of dystrophin.

In conclusion, this report demonstrated the homogeneous distribution of dystrophin in Purkinje neuron somatic and dendritic membranes in both functional cerebellar regions, including the lateral region associated with mental, non-motor abilities, and noted a significantly greater number of puncta in the lateral region compared to the vermal region, implicated in motor aspects of behaviour. The role of dystrophin in the lateral cerebellum, the specific impact of its absence on neuronal function, as in DMD and the *mdx* model, and its relevance to normal cognitive function remain to be elucidated.

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Chapter 3

Experiment 2: Regional Differences in Intrinsic Electrophysiological Properties of Purkinje Neurons from Wild-type and Dystrophin-deficient *mdx* Mice

Objective of Experiment 2: To determine the impact of a lack of dystrophin on the intrinsic electrophysiological properties of Purkinje neurons in the dystrophic brain.

To determine if an absence of brain dystrophin affects intrinsic electrophysiological properties, whole cell patch clamp techniques were employed to examine Purkinje neuron electrophysiology in dissociated cells. Understanding the endogenous properties of Purkinje neurons in which a typically-occurring protein is absent is fundamental to garnering a more complete picture of the consequences of its absence on cerebellar and cognitive functioning. This experiment elucidated the extent to which spontaneous activity within Purkinje neurons is aberrant and could constitute a physiological mechanism to account for cognitive deficits associated with DMD.

Abstract

The protein dystrophin, abundant in muscle, also resides in specific neurons, including cerebellar Purkinje neurons. Although associated with motor behaviours, the cerebellum is thought to play a key role in cognitive processing. In both human Duchenne muscular dystrophy (DMD) and the murine homologue *mdx* model, where muscle and brain dystrophin are absent, aspects of learning and memory are impaired. It is postulated that these impairments are cerebellar-mediated and arise from a lack of Purkinje-neuron dystrophin. Studies thus far examining cerebellar involvement in *md*x mice have not explicitly investigated the lateral cerebellum. This lateral region of the cerebellum is considered to modulate cognitive abilities and possesses distinct circuitry from that of the vermal cerebellum that is associated with motor function. The present study examined the intrinsic electrophysiological properties of Purkinje neurons from both vermal and lateral cerebellum in *mdx* and wild-type (WT) mice. In *mdx* mice, Purkinje neurons exhibited a dramatic reduction in firing regularity and a hyperpolarization of the membrane potential. Firing frequency was also reduced in Purkinje neurons from the lateral cerebellum of *mdx* mice. These findings were specific to the lateral cerebellum of *mdx* mice. Several action potential waveform parameters differed between vermal and lateral Purkinje neurons, irrespective of dystrophin status, including action potential amplitude, slope (both larger in the vermal region), and duration (shorter in the vermal region). Spontaneous action potential firing was also reduced in Purkinje neurons of the vermal region, with membrane hyperpolarization, relative to lateral Purkinje neurons in WT mice. These results suggest an important role for dystrophin in regulating neuronal excitability of Purkinje neurons and support the hypothesis that a loss of cerebellar dystrophin

contributes to cognitive deficits in the *mdx* mouse model and, possibly DMD. Moreover, these results highlight the importance of distinguishing functional zones of the cerebellum in future work characterizing Purkinje neuron electrophysiology and studies using the model of dissociated Purkinje neurons from mice.

3.1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disorder that affects the large human gene, *dystrophin*, resulting in the functional absence of its protein product, dystrophin (Hoffman et al., 1987). In muscle, dystrophin localizes along the sarcolemma where it forms the dystrophin-associated complex (DGC) of proteins (Ervasti et al., 1990). In DMD, the lack of dystrophin and DGC in muscle leads to muscle necrosis (Hoffman et al., 1987) that is ultimately fatal. In addition to the 427 kDa full-length isoform of dystrophin present in muscle (M-type dystrophin), two other promoter-specific full-length isoforms exist in the central nervous system (CNS) (Gorecki et al., 1992); B-type dystrophin is found in pyramidal neurons of the cerebral cortex and hippocampus, and P-type dystrophin resides exclusively in cerebellar Purkinje neurons (Gorecki et al., 1997; Gorecki et al., 1992). Truncated dystrophin isoforms (Dp71, Dp116, Dp140, and Dp260) are also located in the periphery and CNS (as reviewed in Perronnet & Vaillend, 2010).

Cognitive deficits are associated with DMD, and these are attributed to a lack of dystrophin isoforms in the brains of those afflicted (T. W. Kim et al., 1995; Uchino, Teramoto, Naoe, Yoshioka et al., 1994). Among those with DMD, full scale IQ scores follow a normal distribution that is shifted one standard deviation below the population mean, and show a higher prevalence of mental retardation (Cotton et al., 2001). As well, general academic achievement is lower among boys with DMD compared to their unaffected siblings (Hinton et al., 2001; 2004). Specific cognitive deficits are observed in those with DMD; the most consistent finding is a deficit in limited verbal memory, as measured by performance on the Digit Span subtest (S. W. Anderson et al., 1988;

Dorman et al., 1988; Hinton et al., 2000; Leibowitz & Dubowitz, 1981; Ogasawara, 1989; Whelan, 1987). Deficits in immediate verbal memory persist when analysis accounts for general intelligence (Hinton et al., 2000), suggesting a specific cognitive deficit that may translate into difficulties with general intellectual and academic performance. Additional evidence to support the role of dystrophin in cognition comes from studies of *mdx* mice, the murine model of DMD in which both brain and muscle full-length dystrophin isoforms are absent (Uchino, Yoshioka et al., 1994). These mice display cognitive impairments, including deficits in passive-avoidance (Muntoni et al., 1991) and spatial learning (Vaillend et al., 2004) as well as memory deficits (Vaillend et al., 1995).

In the CNS, dystrophin localization is highest in the cerebellum (Lidov et al., 1990) where it is restricted to the cytoplasmic surface of somatic and dendritic membranes of Purkinje neurons. Electrophysiological and molecular studies have identified several perturbations in cerebellar Purkinje neurons of dystrophin-deficient *mdx* mice. At the synaptic level, *mdx* mice exhibit reductions in the inhibitory drive to Purkinje neurons (J. L. Anderson et al., 2003; Kueh et al., 2008; 2011) and in the magnitude of their long-term depression (LTD) response (J. L. Anderson et al., 2004). Immunohistochemical studies demonstrate extensive colocalization of dystrophin and $GABA_A$ neurotransmitter receptor subunits in the postsynaptic membrane (Knuesel et al., 1999). Although absolute levels of $GABA_A$ receptor subunit protein are not affected in the *mdx* mouse brain (Kueh et al., 2008), the number of $GABA_A$ receptor subunits at the postsynaptic membrane is decreased in the *mdx* mouse cerebellum (Knuesel et al., 1999), and the number of extrasynaptic $GABA_A$ receptor subunits is increased (Kueh et al., 2011). These findings strongly implicate Purkinje neuronal dystrophin in $GABA_A$

receptor stabilization and maintenance.

In addition to $GABA_A$ receptor colocalization, dystrophin in the CNS forms a multiprotein DGC similar to that seen in muscle (Waite et al., 2009). The neuronal DGC is implicated in regulating the subcellular localization of proteins central to neuronal excitability, including voltage-gated sodium (Na^+) channels (Gee et al., 1998), inward rectifier potassium (K^+) channels (Connors, Adams, Froehner, & Kofuji, 2004; Leonoudakis et al., 2004), and calcium (Ca^{++}) transporters (J. C. Williams et al., 2006).

Given the strong presence of dystrophin in cerebellar Purkinje neurons relative to other brain regions and the particular cognitive deficits associated with DMD, there is speculation that dystrophin, specifically the lack of P-type dystrophin (herein referred to as "dystrophin") in Purkinje neurons, may account for impaired immediate verbal memory. This speculation is based on the idea that lack of dystrophin would disrupt rehearsal of information in the cerebrocerebellar loops that emanate from the lateral cerebellum (Cyrulnik & Hinton, 2008). Functionally, the mammalian cerebellum can be distinguished as separate functional regions based on their afferent inputs and efferent projections. Located medially, the vermal region projects mainly to the spinal cord and is associated with motor function (Joyal et al., 1996; Nyberg-Hansen & Horn, 1972), whereas the bilateral hemispheres ("lateral cerebellum") receive input from and project to the cerebral cortex (Middleton & Strick, 1997). It is the lateral hemispheres specifically that are implicated in cerebellar-mediated cognition and learning (Allen, Buxton, Wong, & Courchesne, 1997; Decety et al., 1990; Joyal et al., 1996; 2001; Nyberg-Hansen & Horn, 1972; Ryding et al., 1993).

Purkinje neurons constitute the sole output neurons of the cerebellar cortex and

process multiple inputs from multiple brain regions in a consolatory fashion. Purkinje neurons have distinct electrophysiological properties due to the presence of specific ion channels and their conductance properties. In addition to conventional TTX-sensitive transient Na^+ currents, Purkinje neurons possess a TTX-sensitive subthreshold persistent Na^+ current as well as a resurgent Na^+ current that is elicited after initial depolarizations (Raman & Bean, 1997). K^+ channels also encourage high-frequency firing of action potentials by allowing rapid repolarization of Purkinje neurons (Raman & Bean, 1999). Purkinje neurons are also rich in voltage-gated Ca^{++} channels (Llinas, 1988). The unique complement and density of ion channels in the membrane affords Purkinje neurons the ability to fire spontaneous action potentials with high frequency and regularity. This endogenous firing behaviour has been demonstrated in various experimental configurations, including slice preparations using pharmacological blockade of synaptic inputs (Hausser & Clark, 1997; McKay & Turner, 2005), acutely dissociated preparations (Fry, 2006; Nam & Hockberger, 1997; Raman & Bean, 1997; 1999; Raman et al., 1997), cerebellar cultures (Gruol & Franklin, 1987), and *in vivo* recordings of Purkinje neurons (Latham & Paul, 1970; Woodward, Hoffer, & Lapham, 1969).

This "pacemaker" activity of Purkinje neurons is central to intact cerebellar information-processing. This was elegantly demonstrated in a series of experiments by Walter et al. (2006). They showed that in cerebellar slices, pharmalogical blockade of Ca^{++} channels resulted in a decrease in firing regularity in Purkinje neurons in WT mice. In *ducky* mice, a genetic mutation that affects P-type Ca^{++} channels results in decreased Ca^{++} current density, irregular spontaneous Purkinje neuron firing, and ataxia (Walter et al., 2006). In WT mice, whereby pharmalogical blockade impairs Purkinje neuron firing

regularity, and in mutant *ducky* mice, whereby firing irregularity is genetically-driven, firing regularity was restored by EBIO. This compound selectively activates Ca^{++}-activated K^{+} channel type SK and increases its affinity for Ca^{++}, thereby compensating for the decreased Ca^{++} underlying firing irregularity. Moreover, perfusion of EBIO into the cerebellum of *ducky* mice significantly improved motor performance relative to vehicle-treated *ducky* control mice.

Although the cognitive abilities of *ducky* mice have not been examined, learning is impaired in *leaner* mutant mice (Alonso et al., 2008). Similar to *ducky* mice, *leaner* mice also possess a mutation affecting the P-type Ca^{++} channel in Purkinje neurons, resulting in decreased Ca^{++} current density and irregularity of spontaneous firing in these neurons (Walter et al., 2006). These mice show deficits in the Morris water maze, a task assessing spatial learning (Alonso et al., 2008). Although classically considered a hippocampal-dependent learning task (Bannerman et al., 1999), the lateral cerebellum contributes to performance on this task, as evidenced by impairments after lateral cerebellar lesions with no concomitant impairment in motor performance or coordination (Joyal et al., 1996; Joyal et al., 2001). These data demonstrate the importance of intrinsic Purkinje neuron properties in regulating cerebellar function and cerebellar-mediated motor and non-motor processing. Despite evidence documenting the importance of intrinsic Purkinje neuron properties to intact cerebellar function and the suggestion that DMD should be regarded as a disorder of the cerebellum, there are no studies investigating endogenous properties of Purkinje neurons in the dystrophin-deficient brain.

Although dystrophin is typically present in Purkinje neurons of both regions, punctal density is higher in the lateral vs. the vermal region in mice (Snow et al.,

unpublished data, see Chapter 2 in this thesis). Despite the specificity of the contribution from the lateral cerebellum to cognition, studies documenting Purkinje neuron dysfunction in the *mdx* model as a means of investigating DMD-associated cognitive deficits have not examined the lateral region. Moreover, there are no reports comparing the intrinsic membrane properties of Purkinje neurons between the two regions in WT mice. Recently, differences in Purkinje neuron electrophysiology were noted in various lobules of the vermis as a function of their afferent input (i.e. either vestibular or spinal cord) (C. H. Kim et al., 2012). These findings suggest that Purkinje neurons in the lateral cerebellum may differ from those of the vermal region, given the distinct and separate circuitry of these functional zones.

Evidence suggests dystrophin is an integral member of the neuronal postsynaptic apparatus that serves to stabilize ion channels and receptors that drive neuronal excitability. Therefore, we reasoned that a lack of dystrophin in Purkinje neurons would result in dysfunction of their intrinsic electrophysiological properties. We investigated endogenous membrane and firing properties of both vermal and lateral Purkinje neurons in both *mdx* and WT mice. This research aimed at understanding the physiological mechanisms by which cognitive deficits may arise as a result of a lack of dystrophin.

3.2. Materials and Methods

3.2.1. Purkinje Neuron Dissociation

Dystrophic *mdx* mice (n = 20) and WT mice from the same background strain, C57BL10 (n=15) (Jackson Laboratories, Bar Harbor, ME, USA) were used. All procedures were carried out using methods approved by the institutional Animal Care and

Use Protocol Review Committee at the University of Manitoba. The dissociation protocol was modified from that used by Raman and Bean (1997). Mice at postnatal day (P) 16-21 were decapitated, and the head immediately placed in oxygenated Tyrode's solution (in mM: NaCl, 150; KCl, 4; $MgCl_2$, 2; $CaCl_2$, 2; HEPES, 10; and glucose, 10, pH of 7.4 with NaOH). After cooling the head for 20 seconds, superficial vermal or lateral cerebellar tissue was removed and placed into oxygenated cold dissecting solution (DS) (in mM: Na_2SO_4,82; K_2SO_4, 30; $MgCl_2$, 5; HEPES, 10; and glucose, 10, pH of 7.4 with NaOH) for microdissection. Tissue was enzymatically digested in 5 mL of oxygenated DS containing 1.5 mg/mL of protease XXIII (Sigma-Aldrich; Oakville, Ontario) (reduced from 3 mg/mL in original protocol) and 5 mg/ml DNase I (Worthington; Lakewood, New Jersey; an addition to the original protocol) for 6 – 8.5 minutes at 30°C with oxygen blown over the fluid. Tissue was then washed twice in 5 mL of warmed oxygenated DS containing 1 mg/mL of bovine serum albumin (Sigma-Aldrich; Oakvillle, Ontario) and 1 mg/mL of trypsin inhibitor (Sigma-Aldrich; Oakvillle, Ontario) at 30°C, washed once in warmed oxygenated DS at 30°C, and lastly washed in room temperature (RT) Tyrode's solution. Tissue was then triturated in 1.5 mL of Tyrode's solution at RT using fire-polished Pasteur pipettes. Debris was allowed to settle for 1 minute, and the remaining supernatant containing dissociated neurons suspended in solution was plated on glass-bottom culture dishes (MatTek; Ashland, Massachusetts). Purkinje neuron somata were identified by their characteristically large, pear shape and appearance of the primary apical stump of dendrite. Recordings were taken from neurons 30 min to 6 hours after plating at RT.

3.2.2. Electrophysiological Recordings

Patch clamp recordings were carried out using a HEKA EPC10 patch clamp amplifier controlled by Patchmaster 2.54 software (HEKA, Mahone Bay; Nova Scotia). Data were filtered at 2.9 kHz and collected at 10 kHz. Spontaneous and evoked action potentials were recorded from Purkinje neurons in Tyrode's solution (osmolarity of 310-320 mOsm) in current clamp mode using conventional whole-cell techniques. Electrodes were constructed from borosilicate glass with a P-97 Flaming/Brown Pipette Puller (Sutter; Novato, CA). Resistances were 3-8 MΩ when filled with internal recording solution (in mM: K gluconate, 135; NaCl, 10; EGTA, 1.1; HEPES, 10; and MgATP, 5, pH of 7.4 with KOH; osmolarity of 285-290 mOsm). Only recordings in which the series resistance was maintained constant under 30 MΩ were included in the analyses.

Spontaneous action potential recordings

Spontaneous firing was examined in Purkinje neurons for a minimum of two minutes (up to five minutes) without current injection in the current clamp configuration. Action potential frequency and mean membrane potential were measured over two minutes. Mean membrane potential was determined with action potentials removed from the analysis. Regularity of firing was assessed over the same two minutes by calculating the coefficient of variation (CV), defined as the standard deviation of the interspike interval divided by the mean interspike interval.

3.2.3. Evoked Action Potential Properties

Action potentials were evoked from a holding potential of -90 mV using a series of current injections in increments of 10 pA (from -40 pA to +90 pA), 25 pA (from -100

pA to +225 pA), or 50 pA (from -100 pA to +350 pA) (500 ms long each). In order to determine input resistance, voltage-current (V-I) plots were constructed (from traces without action potentials), and the slope of the V-I relationship was calculated. Peak action potential slope was calculated from the peak of the first derivative of the membrane potential. Action potential latency was calculated from the onset of the minimally-sufficient depolarizing current step required to evoke an action potential to the action potential peak. Action potential amplitude was also calculated from the minimally-sufficient current, with amplitude defined as the distance from threshold (see below) to peak spike height. To determine action potential threshold, action potentials were also evoked from a holding potential of -90 mV using a 1-second current ramp (minimum -100 to +100 pA). Threshold was extrapolated from the first derivative of the voltage trace and was defined as the membrane potential at the time point where the first derivative reached 10 mV/ms. To examine action potential firing properties (pattern and frequency) across various membrane potentials, action potentials were evoked from a holding potential of -80 mV using a series of current injections (from -75 pA to +275 pA) in 25-pA steps (10 seconds long each). Membrane potential was taken at the lowest point on the interspike interval (as per Nam & Hockberger, 1997). Data were collected from recorded traces using Spike 2 v5.0 (Cambridge Electronics Design; Cambridge, UK) and OriginPro 8.6 (Microcal; Northampton, Massachusetts).

3.2.4. Statistical Analysis

Data are reported as mean ± SEM. Recordings from Purkinje neurons from each cerebellar region (vermal and lateral; Figure 3.1) were obtained independently from

individual mice. Normality and equality of variances were assessed; for data that met these two assumptions, means were compared using two-way analysis of variance (ANOVA) to determine the significance of the effects of Region (vermal and lateral) x Genotype (WT and *mdx*) and the interaction between those factors. All data met parametric assumptions required for ANOVA except for the frequency of spontaneous firing. In this case, individual planned Mann-Whitney comparisons were conducted on the four individual groups, with the Holm's sequential Bonferroni correction applied to control family-wise error rates. Evoked firing frequency data were collected from current-step protocols. Frequency values were plotted against membrane potential in 5-mV bins ranging from -80 to -45 mV for each Purkinje neuron. The resulting plots were fitted to sigmoidal curves using nonlinear regression using the function $y = A2 + (A1-A2) / (1 + \exp((x-x0)/dx))$, where A1, corresponding to the initial frequency value, was fixed at 0. Individual planned comparisons of entire curves were conducted on the four individual groups using F-tests, with Holm's sequential Bonferroni correction applied. Statistical analyses were conducted using OriginPro 8.6 (Microcal; Northampton, Massachusetts) and Excel 2010. With the exception of Bonferroni sequentially-corrected comparisons, statistical significance was set at $p < 0.05$.

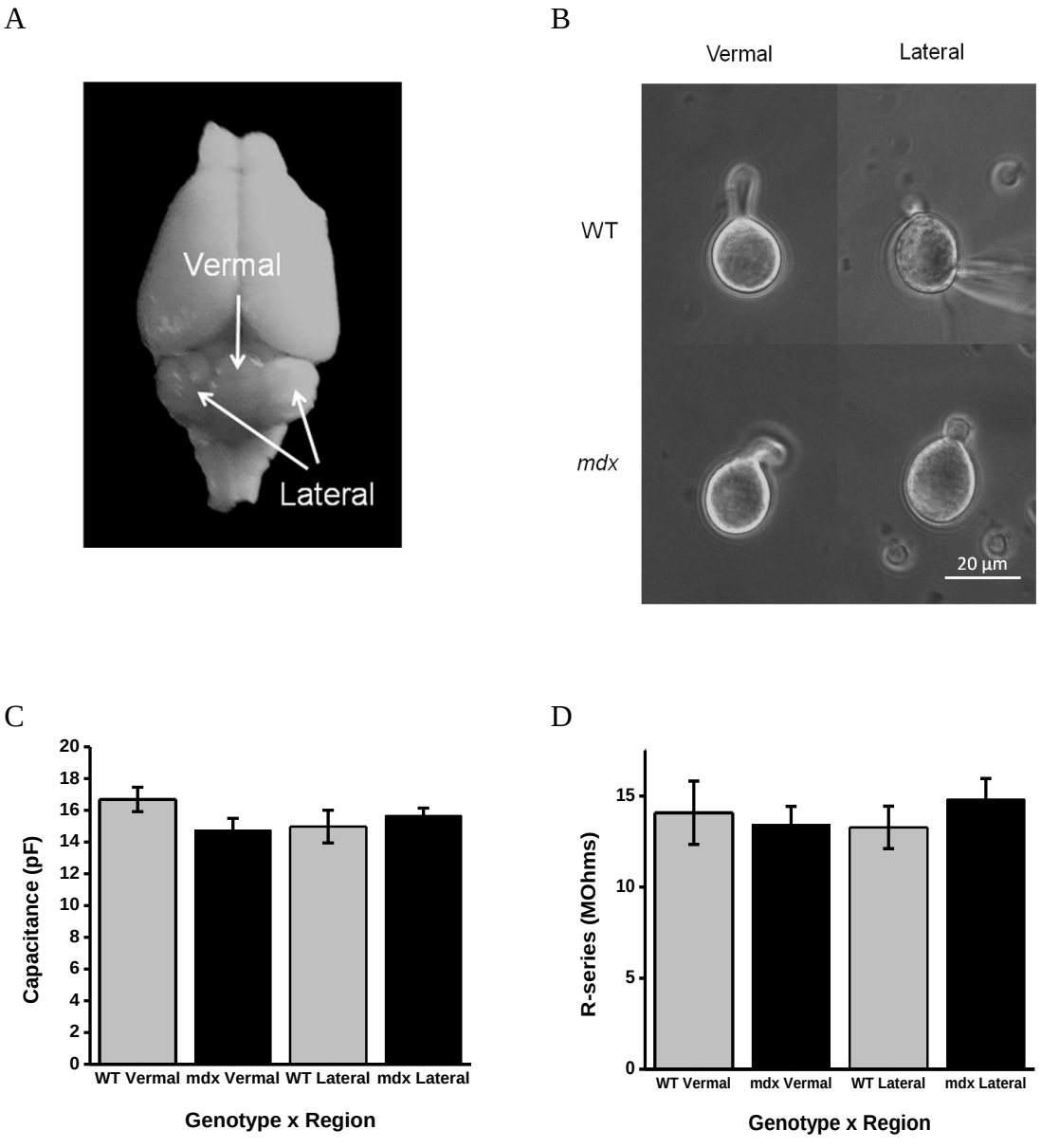
3.3. Results

3.3.1. Recording Parameters

Current clamp recordings were obtained from a total of 55 WT (vermal: n = 14; WT lateral: n= 12) and *mdx* (vermal: n = 13; lateral: n = 16) Purkinje neurons (Figure 3.1B). There were no obvious differences in the number of cells that were amenable to

whole-cell patch clamping between Purkinje neurons from *mdx* and WT mice, although this was not quantified. Cell capacitance, an indicator of cellular membrane area, was not significantly different between region ($F_{(1,51)} = 0.29$, $p = 0.59$) or genotype ($F_{(1,51)} = 0.68$, $p = 0.41$), nor was there a significant interaction between the two factors ($F_{(1,51)} = 3.11$, $p = 0.08$; grand mean = 15.56 pF ± 0.37, N = 55; Figure 3.1C). As series resistance in recordings can affect the action potential parameters under investigation (i.e., amplitude), the mean series resistance across groups was also subjected to a two-way ANOVA. There were no significant differences in this parameter, either between regions ($F_{(1,51)} = 0.05$, $p = 0.83$) or genotypes ($F_{(1,51)} = 0.12$, $p = 0.73$), nor was there a significant interaction effect ($F_{(1,51)} = 0.69$, $p = 0.4$; grand mean = 13.97 ± 0.64 MΩ, N = 55; Figure 3.1D).

Figure 3.1. (A) Mouse brain illustrating regional divisions in the cerebellum. (B) Phase contrast images (40x) of representative dissociated Purkinje neurons from WT and *mdx* vermal and lateral cerebellum, with viable neurons appearing phase-bright. (C) Mean cell capacitance, an indicator of neuronal membrane area, was similar across all groups, as analyzed by a two-way ANOVA (data shown for individual groups). (D) Mean series resistance in recordings was also similar across groups (two-way ANOVA; data shown for individual groups). N=55 Purkinje neurons (WT vermal: n = 14; WT lateral: n= 12; *mdx* vermal: n = 13; *mdx* lateral: n = 16 for C and D).



3.3.2. Evoked Recording Parameters

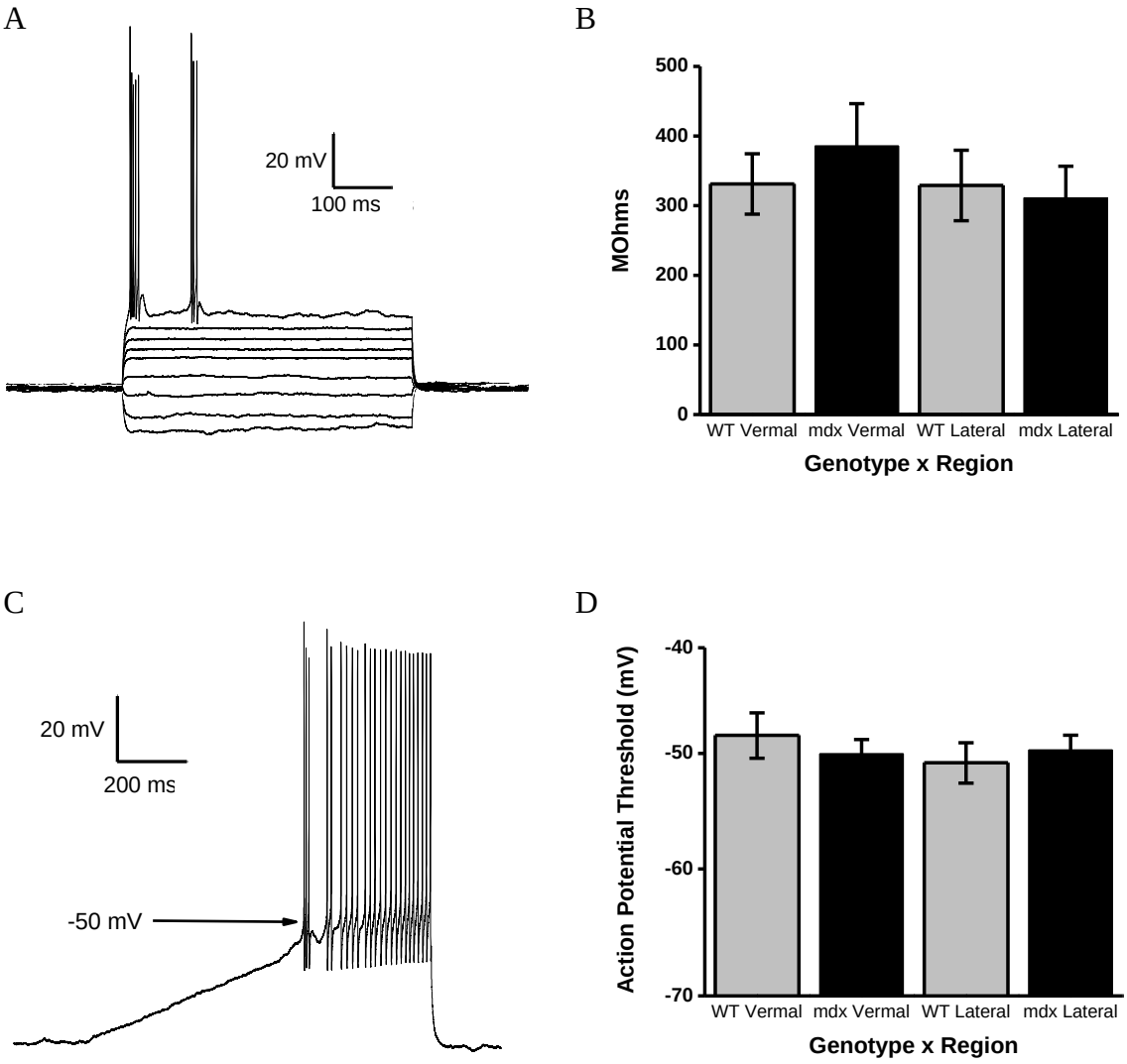
3.3.2.1. Input resistance

To examine input resistance, changes in membrane potential as a function of injected current were calculated using a series of current steps (Figure 3.2A). The mean input resistance of Purkinje neurons did not differ between region ($F_{(1,51)}$ = 0.58, p = 0.45; vermal = 357.48 ± 36.58 MΩ, n = 27; lateral = 318.88 ± 33.24 MΩ, n = 28) or genotype ($F_{(1,51)}$ = 0.13, p = 0.72; WT = 330.21 ± 32.36 MΩ, n = 26; *mdx* = 344.66 ± 37.00 MΩ, n = 29). There was no significant interaction between region and genotype ($F_{(1,51)}$ = 0.52, p = 0.48). Therefore, the input resistance of Purkinje neurons was found to be similar across all groups (grand mean = 337.83 ± 24.65 MΩ, N = 55) (Figure 3.2B).

3.3.2.2. Threshold

Using a 1-second depolarizing current ramp (Figure 3.2C), no significant differences were found related to region ($F_{(1,51)}$ = 0.42, p = 0.52; vermal = -49.16 ± 1.24 mV, n = 27; lateral = -50.18 ± 1.08 mV, n = 28) or genotype ($F_{(1,51)}$ = 0.02, p = 0.88; WT = -49.50 ± 1.4 mV, n = 26; *mdx* = -49.84 ± 0.93 mV, n = 29). As well, no significant interaction effect was found ($F_{(1,51)}$ = 0.73, p = 0.4), indicating that action potentials in Purkinje neurons from vermal and lateral WT and *mdx* cerebellum were evoked at similar membrane potentials (grand mean = -49.68 ± 0.81 mV; N = 55) (Figure 3.2D).

Figure 3.2. (A) Membrane potential traces from a representative Purkinje neuron from *mdx* lateral cerebellum that was subjected to a current-step protocol. Purkinje neurons were briefly held at -90 mV, followed by application of a series of hyperpolarizing, then depolarizing pulses in increasing increments of 10 pA. Input resistance was defined as the slope of the line fitted with the change in membrane potential over amplitude of current injected. (B) The input resistance of Purkinje neurons was similar across all groups (two-way ANOVA; data shown for individual groups). (C) Trace from a representative Purkinje neuron from WT vermal cerebellum that was subjected to a ramp protocol. After holding the Purkinje neuron briefly at -90 mV, a 1-second current ramp was applied (minimum -100 pA to +100 pA). (D) In evoked action potentials, the firing threshold (defined as a 10 mV/ms rate of change in the slope of the first action potential) was similar across all groups (two-way ANOVA; data shown for individual groups). N=55 Purkinje neurons (WT vermal: n = 14; WT lateral: n= 12; *mdx* vermal: n = 13; *mdx* lateral: n = 16 for B and D).



3.3.2.3. Waveform properties differ between cerebellar regions.

Several evoked action potential waveform properties were examined to investigate region and/or genotypic differences. These parameters included action potential amplitude, rate of depolarization (slope), duration, and latency. Data were gathered from the first action potential evoked from a minimally-sufficient current step (as in Figure 3.2A).

3.3.2.3.1. Action Potential Amplitude

The amplitude of the first evoked action potential in a series of current steps was significantly higher ($F_{(1,51)} = 9.17$, $p = 0.004$) in Purkinje neurons from the vermal (92.74 ± 1.67 mV, n = 27) than the lateral region (85.00 ± 2.00 mV, n = 28). The main effect of genotype was not significant ($F_{(1,51)} = 1.38$, $p = 0.25$; WT = 87.54 ± 2.20 mV, n = 26; *mdx* = 89.92 ± 1.78 mV, n = 29). No significant interaction effect was found ($F_{(1,51)} = 0.02$, $p = 0.9$), indicating that the increased amplitude in vermal Purkinje neurons was consistent regardless of genotype (Figure 3.3A-B).

3.3.2.3.2. Action Potential Slope

As with amplitude, there was a significant effect of region in the rate of depolarization during an action potential, as measured by the slope of the first evoked action potential ($F_{(1,51)} = 8.66$, $p < 0.005$), but again not for genotype ($F_{(1,51)} = 1.05$, $p = 0.3$; WT = 413.20 ± 22.28 mV/ms, n = 26; *mdx* = 434.30 ± 19.44 mV/ms, n = 29) nor the interaction between the two factors ($F_{(1,51)} = 0.01$, p = 0.9). Action potential slope was

significantly higher in vermal (464.80 ± 17.51 mV/ms, n = 27) than lateral Purkinje neurons (385.29 ± 21.00 mV/ms, n = 28), regardless of genotype (Figure 3.3C-D).

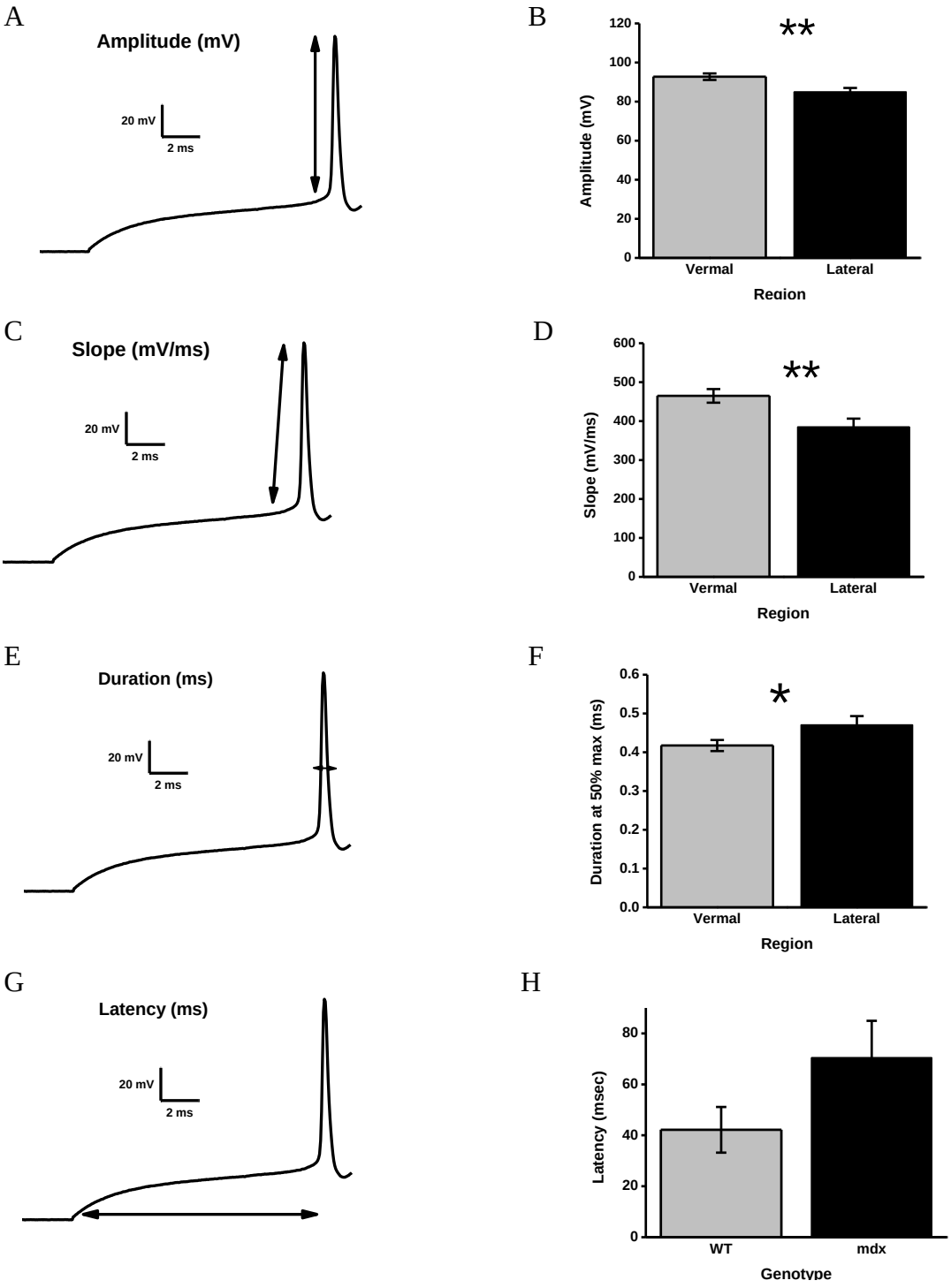
3.3.2.3.3. Action Potential Duration

There was a significant effect of region ($F_{(1,51)}$ = 4.52, p = 0.038) without an interaction effect ($F_{(1,51)}$ = 1.23, p = 0.27) for the duration of the first action potential evoked with a minimally-sufficient current step, measured at 50% of the maximum amplitude. The effect of genotype was not significant for this parameter ($F_{(1,51)}$ = 0.72, p = 0.25; WT = 0.45 ± 0.02 ms, n = 26; *mdx* = 0.44 ± 0.02 ms, n = 29). These results indicate that, irrespective of genotype, action potentials were significantly shorter in duration in vermal (0.42 ± 0.01 ms, n = 27) than lateral (0.47 ± 0.02 ms, n = 28) Purkinje neurons (Figure 3.3E-F).

3.3.2.3.4. Latency

Analysis of the latency from the onset of the minimally-sufficient depolarizing current step to the peak of the first action potential did not reveal any significant differences between regions ($F_{(1,51)}$ = 0.06, p = 0.8; vermal = 58.40 ± 11.81 ms, n = 27; lateral = 55.87 ± 13.36 ms, n = 28). Although the latency to peak action potential height was greater in *mdx* Purkinje neurons (70.50 ± 14.45 ms, n = 29) than in WT Purkinje neurons (42.17 ± 8.96 ms, n = 26), this difference fell short of statistical significance ($F_{(1,51)}$ = 2.67, p = 0.1) (Figure 3.3G-H). There was no significant interaction between region and genotype ($F_{(1,51)}$ = 0.87, p = 0.36).

Figure 3.3. Regional differences in waveform properties of action potentials in Purkinje neurons. (A, C, E, G) Schematics of waveform properties of the first evoked action potential. (B) Action potentials of Purkinje neurons from the vermal cerebellum (regions combined) were higher in amplitude and (D) slope. (F) Vermal Purkinje neurons (n = 27) fired shorter-lasting action potentials than lateral (n = 28) Purkinje neurons (genotypes combined). (H) There was a trend ($p = 0.1$) towards an increased latency in *mdx* (n = 29) vs. WT (n = 26) Purkinje neurons (regions combined). *$p < 0.05$, **$p < 0.01$.



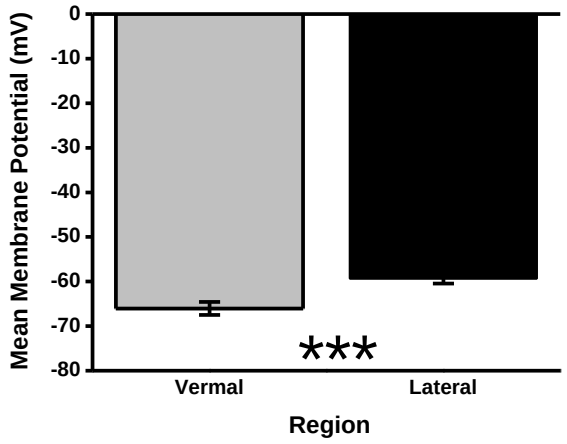
3.3.3. Spontaneous and Evoked Action Potential Firing Frequency and Pattern

3.3.3.1. Dystrophin-deficiency hyperpolarizes the membrane potential, reduces spontaneous firing frequency, and inhibits tonic firing.

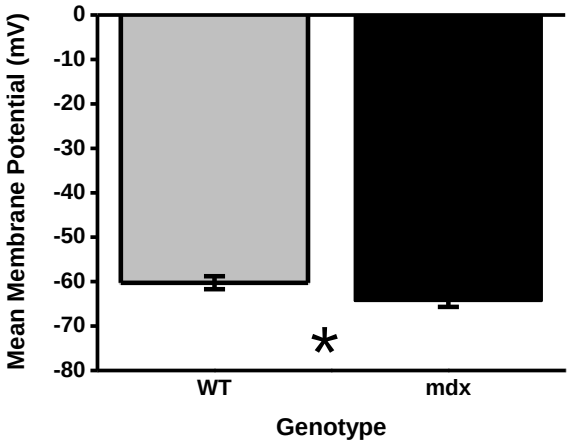
After break-in and stabilization of the cell in the whole-cell configuration in current clamp mode, Purkinje neuron action potential activity was assessed without current injection. Mean membrane potentials were significantly different as a function of region ($F_{(1,51)} = 15.94$, $p < 0.001$) and genotype ($F_{(1,51)} = 6.45$, $p < 0.05$), with no significant interaction effect ($F_{(1,51)} = 0.11$, $p = 0.7$). The mean membrane potential of Purkinje neurons from the vermal region was hyperpolarized relative to those from the lateral region (-66.05 ± 1.45 mV, n = 25; -59.34 ± 1.13 mV, n = 28, respectively; $p < 0.001$), as were those from *mdx* mice relative to WT mice (-64.37 ± 1.33 mV, n = 29; -60.25 ± 1.46 mV, n = 24, respectively; $p < 0.05$) (Figure 3.4).

Figure 3.4. (A) Mean membrane potentials were hyperpolarized in Purkinje neurons from the vermal (n = 25) vs. the lateral (n = 28) region and (B) in those from *mdx* (n = 29) vs. WT (n = 24) mice. **p* < 0.05, ****p* < 0.001.

A



B



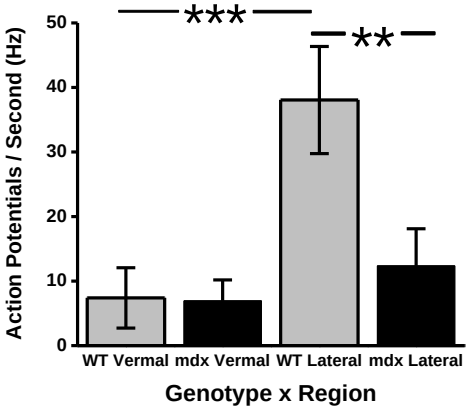
Spontaneous firing frequency data did not meet assumptions required for analysis using a two-way ANOVA. Therefore, individual Mann-Whitney (MW) pairwise comparisons with Bonferroni sequential correction were conducted. This analysis revealed a dramatically lower firing frequency in vermal (7.39 ± 4.67 Hz, n = 12) compared to lateral Purkinje neurons (38.05 ± 8.31 Hz, n = 12) in WT mice

(MW: $p = 0.0008$). This regional difference, however, fell short of statistical significance (MW: $p = 0.06$) between *mdx* Purkinje neurons from the lateral (12.36 ± 5.74 Hz, n = 16) and vermal region (6.95 ± 3.23 Hz, n = 13). There was a sharp decrease in firing frequency in Purkinje neurons from *mdx* mouse lateral cerebellum (12.36 ± 5.74 Hz) compared to WT neurons from the same region (38.05 ± 8.31 Hz, MW: $p = 0.003$). Firing frequency, however, was not different (MW: $p = 0.5$) between vermal Purkinje neurons from WT and *mdx* mice (7.39 ± 4.67 Hz; 6.95 ± 3.23 Hz, respectively) (Figure 3.5A).

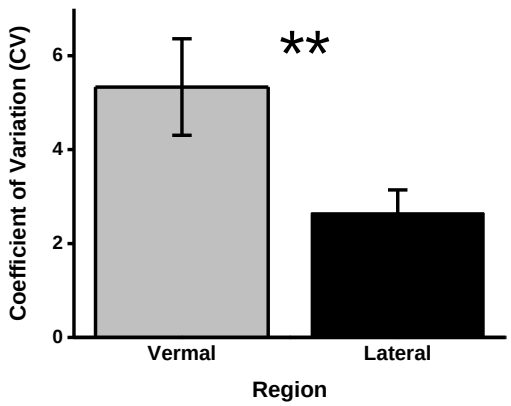
Firing pattern was assessed during the same two-minute period in spontaneously active Purkinje neurons by calculating the CV. A larger CV indicates more irregular spiking patterns, whereas a lower CV is indicative of more regular, tonic firing. As with mean membrane potential, significant main effects of region ($F_{(1,51)} = 9.72$, $p = 0.003$) and genotype ($F_{(1,51)} = 17.84$, $p = 0.0001$) were detected, with no significant interaction between the two factors ($F_{(1,51)} = 1.63$, $p = 0.2$). Purkinje neurons from *mdx* mice (CV = 5.47 ± 0.85, n = 27) and those from the vermal region (CV = 5.33 ± 1.03, n = 22) exhibited roughly two-fold larger CVs and, hence, more irregular firing than WT Purkinje neurons (CV = 1.91 ± 0.43, n = 23) or those from the lateral region (CV = 2.65 ± 0.49, n = 28) (Figure 3.5B & C). The group differences noted in the CV parallel the differences in hyperpolarization of the membrane potential across the groups examined.

Figure 3.5. Mean spontaneous action potential firing frequency (A) and regularity (B and C) in dissociated Purkinje neurons. (A)In WT mice, vermal Purkinje neurons (n = 12) exhibited less spontaneous firing than lateral Purkinje neurons (n = 12). Firing frequency was also reduced in lateral Purkinje neurons from *mdx* (n = 16) vs. WT (n= 12) mice. (B) Action potential firing was more irregular in Purkinje neurons from vermal (n = 22) vs. lateral (n = 28) cerebellum and (C) in Purkinje neurons from *mdx* mice (n = 27) compared to WT (n = 23). $^{**}p < 0.01$, $^{***}p < 0.001$.

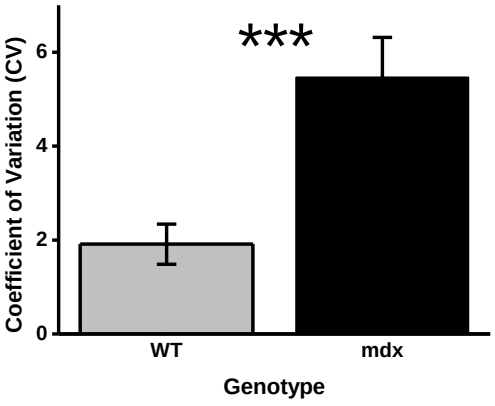
A



B



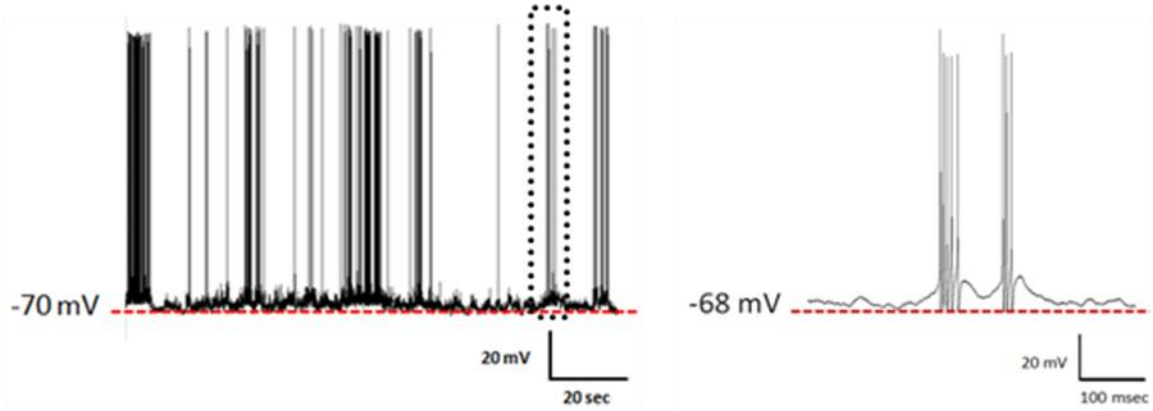
C



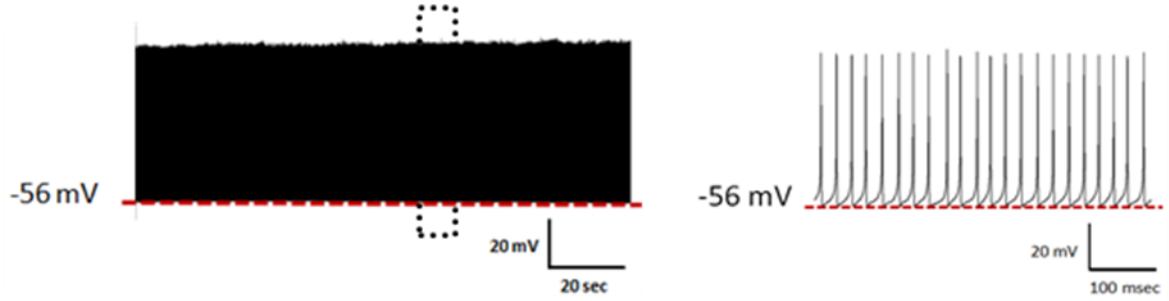
Qualitatively, clear differences emerged when examining firing pattern between groups and regions. In the vermal region, Purkinje neurons from both WT and *mdx* mice displayed tonic firing in only a small subset of neurons (WT: 3/12, 25%; *mdx*: 3/13, 23.1 %), with a nearly identical occurrence of tonic-firing cells in each group. Rather, these neurons often displayed a bursting pattern of spontaneous activity, with periods of quiescence in between bouts of action potentials (Figure 3.6A). This is in sharp contrast to Purkinje neurons from the WT lateral cerebellum, where most (10/12; 83.3%) exhibited sustained tonic, repetitive firing (Figure 3.6B). Less than half the Purkinje neurons (7/16; 43.75%) from the *mdx* mouse lateral cerebellum, however, displayed tonic firing, and often exhibited burst firing (Figure 3.6C). Therefore, Purkinje neurons from *mdx* mice did not display the regional differences (prevalence for bursting in the vermal region and tonic firing in the lateral region) that were observed in WT mice. By comparison, Purkinje neurons from both regions of the dystrophic mouse brain displayed a high degree of bursting behaviour. These data are consistent with the findings of reduced action potential frequency as a function of genotype in the lateral region only, and as a function of region in WT mice.

Figure 3.6. (A-C) *Left.* Membrane potential trace representing two-minutes of spontaneous activity in dissociated Purkinje neurons in WT and lateral region in *mdx* mice in current clamp mode (no current applied). Purkinje neurons from both vermal and lateral regions in *mdx* mice typically displayed burst firing, therefore trace shown from lateral cerebellum only in *mdx* mice. *Right.* Expanded scale of recording taken from portion (500 ms) of boxed area. (A) Burst firing in Purkinje neuron from the WT vermal cerebellum. (B) This is in sharp contrast to the tonic, rhythmic firing pattern often exhibited by lateral Purkinje neurons in the WT cerebellum. (C) Unlike lateral Purkinje neurons from WT mice, lateral Purkinje neurons from *mdx* mice often displayed bursting behaviour, consistent with spontaneous activity in the vermal cerebellum in WT mice.

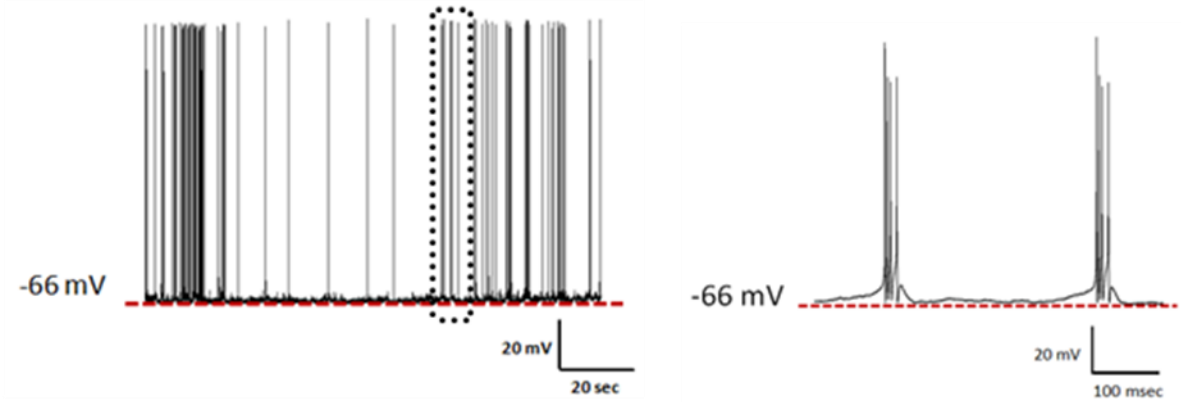
A WT Vermal



B WT Lateral



C *mdx* Lateral

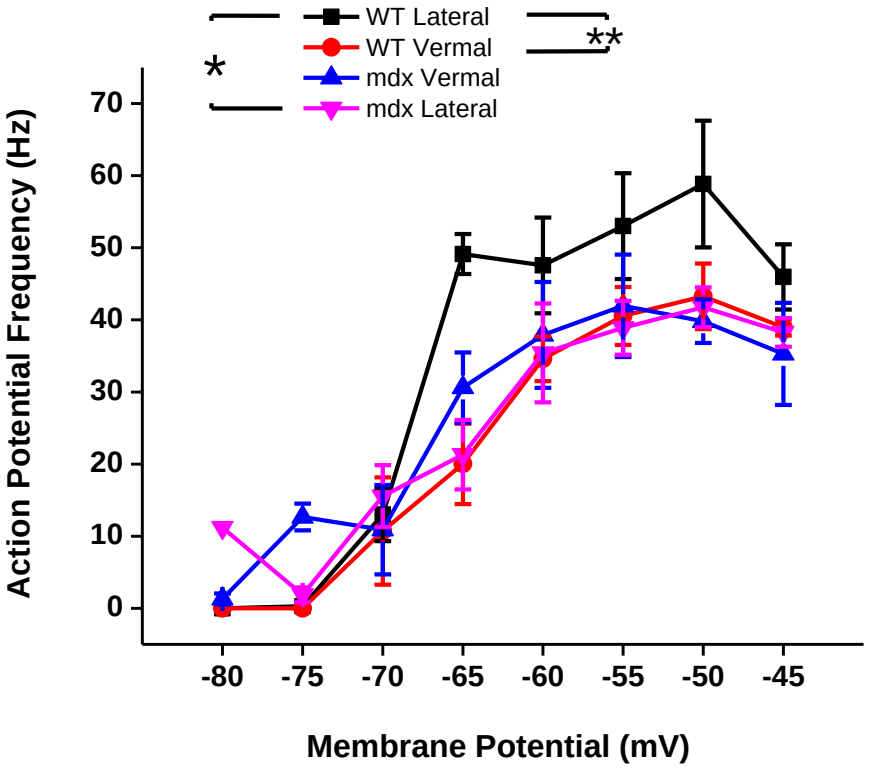


Previous research has reported an inability of Purkinje neurons to fire tonically in mice with mutations that down-regulate voltage-gated Na^+ channels (Shakkottai et al., 2009). To determine if Purkinje neurons that did not fire in a tonic fashion (i.e., bursting or silent Purkinje neurons) in the present study could be evoked to do so, depolarizing current steps were applied for 10 seconds from a holding potential of -80 mV. In 100% of the 26 Purkinje neurons examined for which there were data (WT Vermal: data on 8 of 8 Purkinje neurons that did not exhibit tonic firing; WT lateral: 2 of 2; *mdx* vermal: 9 of 10; and *mdx* lateral: 7 of 9) these depolarizing steps were sufficient to induce tonic firing (data not shown). This finding demonstrates that despite the regional and genotypic differences noted in spontaneous excitability, all Purkinje neurons had the capacity to fire repetitively when stimulated.

In addition to investigating firing pattern with stimulation, firing frequency was also examined in Purkinje neurons with stimulation using depolarizing current steps from a holding potential of -80 mV. Frequency data were plotted as a function of membrane potential. In all groups, firing frequency increased as the membrane potential became more depolarized (Figure 3.7). Action potential failure became evident in all groups at membrane potentials depolarized more than -50 mV. The relationship between membrane potential and firing frequency, however, was significantly different in Purkinje neurons from WT lateral cerebellum (n= 10), where stimulation to similar membrane potentials resulted in increased firing frequency relative to Purkinje neurons from either WT vermal ($p < 0.0001$; n = 6) or *mdx* lateral cerebellum ($p < 0.001$; n = 12). This increased firing in WT lateral Purkinje neurons occurred across a range of membrane potentials, with a particularly sharp increase in firing frequency between -70 mV and -65 mV. These

regional differences in the frequency-mV relationship were not detected between *mdx* vermal (n=11) and lateral cerebellum (p = 0.76; n = 12), nor were there genotypic differences in frequency-mV relationship for the vermal region (p = 0.18). Examination of evoked firing is consistent with data from spontaneous recordings, as in both parameters, Purkinje neurons from the lateral region in WT mice displayed generalized increased firing levels relative to those from the vermal region, but this regional difference was eliminated in Purkinje neurons from *mdx* mice.

Figure 3.7. Plot of evoked firing frequency against membrane potential. Firing frequency was calculated over 10 seconds with 25-pA current steps. Membrane potentials were binned in 5-mV increments, plotted, and fitted with a sigmoidal curve for each of the four groups. Consistent with spontaneous firing behaviour, Purkinje neurons from WT lateral cerebellum (n = 10) exhibited consistently higher firing rates at various membrane potentials relative to those from the vermal region in WT (n = 6) or in the lateral in *mdx* mice (n = 12). As with spontaneous firing, the frequency of evoked firing did not display a regional difference in *mdx* mice as was seen in WT mice. *p < 0.001, **p < 0.0001.



3.4. Discussion

The purpose of this study was to investigate the impact of a loss of dystrophin using the *mdx* mouse model of DMD on the electrophysiological properties of Purkinje neurons whilst taking cerebellar functional zone into account. To our knowledge, this is the first study to explicitly examine the intrinsic membrane properties of Purkinje neurons from the lateral cerebellum, associated with cognition, in either the typical brain or in *mdx* dystrophic mice. These experiments revealed several action potential waveform parameters of Purkinje neurons that differed between cerebellar functional zones. As well, the rate of spontaneous and evoked activity differed regionally in WT mice, with elevated firing rate in the lateral relative to the vermal region. This regional elevation, however, was not seen in Purkinje neurons from *mdx* mice, as the rate of spontaneous and evoked action potential activity was drastically reduced in *mdx* mouse Purkinje neurons from the lateral region relative to WT Purkinje neurons from the same region. This is an intriguing finding, as the lateral region, associated with cognition, also displays a higher density of dystrophin (Snow et al., unpublished, see Chapter 2). Mean membrane potential and firing regularity were both significantly altered from WT mouse Purkinje neuron properties in *mdx* mice, regardless of region. Action potential shape was unaffected by a lack of dystrophin in Purkinje neurons. Of particular importance is the observation that there are regional differences in intrinsic membrane properties of Purkinje somata between cerebellar functional zones in typical mice that are abolished in mice lacking dystrophin. These findings support the notion that a loss of cerebellar dystrophin may contribute to cognitive deficits in the *mdx* model and DMD.

3.4.1. Regional Differences

3.4.1.1. Evoked Responses

Although this is the first study to report differences in Purkinje neuron intrinsic membrane properties between vermal and lateral cerebellum, a recent study found lobule-specific differences in action potential waveform properties in vermal cerebellar Purkinje neurons (C. H. Kim et al., 2012). Both amplitude and rate of action potential upstroke were significantly greater in Purkinje neurons from lobules III-IV, part of the spinocerebellum, relative to those from lobule X, a component of the vestibulocerebellum. Our comparison of evoked action potential characteristics between vermal and lateral Purkinje neurons extends these findings to include distinctive Purkinje neuron properties in cerebellar regions involved in cognition. We noted broader, smaller-amplitude waveforms in lateral relative to vermal Purkinje neurons, properties that are largely Na^+-mediated. The results may suggest there are corresponding regional differences in Na^+ channel localization, density, and/or kinetics in Purkinje neuron somata.

Purkinje neurons possess a diversity of Na^+ channels and accompanying currents that dictate action potential shape and firing rates, including transient, persistent, and resurgent Na^+ currents. In dissociated mouse Purkinje neurons, the development of large-amplitude, fast-rising action potentials by P12 coincided with increased density of all three types of Na^+ current (Fry, 2006). Of note also is the present observation that Na^+-mediated action potential threshold was not different between regions. In Purkinje neurons, Na^+-channel availability at the soma is a strong determinant of action potential threshold in Purkinje neurons (Khaliq & Raman, 2006). Although the findings of regional

differences in action potential waveforms suggest corresponding regional differences in properties of these three current types, their relative contributions to the parameters studied here are unknown.

Regional differences in Na^+ conductances are expected to reflect waveform differences and may also contribute to the differences in spontaneous firing behaviour noted in WT mice. Several studies have established the importance of Na^+ current in high-frequency firing (Raman & Bean, 1999), as application of TTX, which blocks Na^+-channel activation, abolishes firing. TTX-sensitive resurgent Na^+ current also contributes to Purkinje neuron excitability, allowing for subsequent action potentials to be fired in quick succession by contributing substantial Na^+ current during the interspike interval (Raman & Bean, 1997). Spontaneous activity of Purkinje neurons is diminished in *med* mice, in which Nav1.6 channel expression that contributes substantially to resurgent Na^+ current is absent (Raman et al., 1997). Nav1.6-deficient Purkinje neurons have been shown to be incapable of high-frequency firing, even with stimulation (Shakkottai et al., 2009). Although all Purkinje neurons in the present study fired tonically (either spontaneously or with stimulation), a generalized decrease in action potential firing (both spontaneous and evoked) was noted in vermal compared to lateral Purkinje neurons, further implicating Na^+ conductances in regional specialization. Despite decreased firing frequency in vermal Purkinje neurons, which is associated with decreased Na^+ conductance (Raman et al., 1997; Shakkottai et al., 2009; Fry, 2006), evoked action potentials in vermal Purkinje neurons were narrower, larger in amplitude, and faster-rising relative to those from the lateral region. Such a waveform suggests *increased* Na^+ conductance in vermal Purkinje neurons, as the appearance of narrow, large action

potentials mirrors the development time course of Na^+- current upregulation (Fry, 2006). Investigation of individual ionic currents in voltage-clamp experiments could provide insight into the relative contributions of the various Na^+ currents (i.e. transient, persistent, and resurgent) to the noted regional differences in Purkinje neuron action potential properties. As well, studies of Na^+ channel gene expression using real-time polymerase chain reaction could determine if there are corresponding regional differences in ion channel expression that could account for the noted regional variations in intrinsic membrane properties seen in Purkinje neurons.

It must be noted that in addition to the contribution of Na^+-channel conductances to action potential shape and firing frequency, K^+ channels also play a major role in Purkinje neuron function. Specifically, the presence of Kv3 channels is associated with high-frequency firing in Purkinje neurons (Akemann & Knopfel, 2006). Given the diversity of ion-channel distribution and conductance properties and the considerable control they exert over Purkinje neuron physiology, results of the present study merit a regional investigation into ion-channel properties to further understand the complex input-output relationships of Purkinje neurons.

3.4.1.2. Patterns of Spontaneous Purkinje Neuron Activity between Regions

Results of the present study indicate that a lack of dystrophin (as in *mdx* mouse cerebellum) or a reduction in dystrophin (as in the WT vermal vs. lateral cerebellum) is associated with a decrease in spontaneous firing frequency, and an increased propensity for bursting versus high-frequency tonic firing (in lateral WT Purkinje neurons). Although Purkinje neurons have long been known to exhibit tonic firing (Fry, 2006; Fry,

Boegle, & Maue, 2007; Gruol & Franklin, 1987; Hausser & Clark, 1997; Raman & Bean, 1997; 1999; Raman et al., 1997), burst firing occurs in Purkinje neurons in slices, where it constitutes one of the features in a trimodal pattern of firing seen in this preparation (McKay & Turner, 2005; Womack & Khodakhah, 2002). In dissociated preparations, roughly 20% of isolated Purkinje neurons fire spontaneous bursts (Swensen & Bean, 2003), similar to the proportion found in the present study in WT lateral cerebellum. No mention is made, however, of the particular cerebellar region examined in the study by Swensen and Bean, making any direct comparisons with the present study difficult. Burst firing is often seen at hyperpolarized membrane potentials relative to that required for tonic firing (Swensen & Bean, 2003), and this is consistent with our results showing a hyperpolarization of the membrane potential and a tendency for bursting in WT vermal cerebellum and in both regions of the *mdx* mouse cerebellum.

Kim et al. (2012) also noted differences in evoked action potential firing pattern based on location within the vermal region. In Purkinje neurons from lobules III-IV, 68% of Purkinje neurons displayed tonic firing upon stimulation, whereas only 27% of Purkinje neurons from lobule X fired in this fashion. In lobule X, firing pattern was more diverse than in other lobules, and included classical repetitive bursting as well as initial bursting. In initial bursting, Purkinje neurons fired a single burst followed by quiescence during the remainder of the current pulse. Tonic firing could not be initiated, regardless of the stimulus intensity, in the study by Kim and colleagues. Gap firing was also reported in this lobule, in which there was a long delay between the onset of the stimulus and the first action potential. This pause between stimulus and response was removed with additional current. Neither firing pattern was detected in the present study, as the

vermal lobules that were sampled were isolated from the dorsal aspect of the cerebellum (as they were easily accessible upon removal of the skull), and are thus, likely to correspond to Purkinje neurons in lobules VI-IX. Reports of heterogeneous firing pattern in Purkinje neurons between vermal lobules in the study by Kim et al. (2012) and our findings of a propensity for burst firing in the vermal cerebellum and for tonic firing in the lateral cerebellum indicate a diversity of action potential firing across functional zones of the cerebellum that is only beginning to be appreciated.

The cerebellum is often considered an information-processing "machine" (Eccles et al., 1967; Ito, 2006), with Purkinje neurons serving as the major processing unit. As local neuronal circuitry is preserved within regions of the cerebellum, so is the manner in which information is processed (Ito, 2006). Thus, the cerebellar contribution to both motor and non-motor skills arises from its interconnections with other brain regions (Leiner et al., 1986; 1989). Region- and lobule-specific differences in endogenous behaviour of Purkinje neurons may represent fine-tuning at the cellular level (through ion-channel regulation) that has developed to optimize processing of the specific inputs under their jurisdiction.

3.4.2. Genotypic Differences

Studies have documented GABA-dependent alterations in synaptic transmission of *mdx* Purkinje neurons, including a reduction in frequency and amplitude of miniature inhibitory postsynaptic currents (Kueh et al., 2008; 2011), consistent with the findings of decreased $GABA_A$ receptor clustering in *mdx* Purkinje neurons (Knuesel et al., 1999). Studies examining the effects of GABA on Purkinje neuron activity demonstrate its

ability to modulate spontaneous Purkinje neuron behaviour. In WT mice, blockade of $GABA_A$ receptor-induced inhibition in cerebellar slices results in increased spontaneous firing frequency and regularity in Purkinje neurons (Hausser & Clark, 1997). Focal application of GABA to Purkinje neuron somata results in membrane hyperpolarization and diminished spontaneous firing (Okamoto, Kimura, & Sakai, 1983), similar to present findings in *mdx* mice. Given these studies, coupled with the reported perturbations in $GABA_A$ receptor clustering in and resulting decreased inhibition of *mdx* Purkinje neurons, it is reasonable to speculate that the present results reflect the consequences of GABA-ergic alterations in *mdx* mice. Reduced firing rates and membrane hyperpolarization, however, occur after an increase in the inhibitory effects of GABA at the soma (*ibid*). The reduced inhibition of Purkinje neurons in *mdx* would be expected to induce *increased* spontaneous activity and membrane *depolarization*, in contrast to the present findings. Moreover, the perturbations in intrinsic membrane properties reported here in the *mdx* model are presumed to be GABA-independent, as all synaptic inputs are eliminated in the dissociated preparation, and GABA was not included in the external solution. Other studies have also demonstrated GABA-independent perturbations in *mdx* Purkinje neurons, including diminished LTD (J. L. Anderson et al., 2004) and a reduction in the inhibitory drive to Purkinje neurons upon stimulation of the molecular layer (J. L. Anderson et al., 2003), as in both studies, GABA-ergic input was pharmacologically blocked. Thus, although GABA-mediated activity is clearly affected in Purkinje neurons lacking dystrophin, other mechanisms of pathology are beginning to be elucidated.

The hyperpolarization in membrane potential and reduction in firing frequency and regularity in Purkinje neurons from *mdx* mice lacking dystrophin suggest that in

addition to GABA-receptor stabilization, dystrophin may be integral in the maintenance of other proteins central to regulating membrane potential. In studies of synaptic transmission in *mdx* mice (J. L. Anderson et al., 2003; J. L. Anderson et al., 2010), membrane potential was not significantly different in *mdx* Purkinje neurons relative to controls. It is worth noting, however, that the mean membrane potential of *mdx* Purkinje neurons was hyperpolarized relative to controls in both previous reports but failed to reach statistical significance, with between-group mean differences similar in magnitude to those reported here. Thus, the inconsistency in findings between those studies and the current study is most likely due to the use of smaller sample sizes, and hence reduced statistical power, in previous reports.

In whole-cell recordings from Purkinje neuron somata, pharmacological blockade of I_h, a hyperpolarized-induced cation current, results in periods of quiescence in normally tonic-firing Purkinje neurons with a concomitant membrane hyperpolarization. This suggests that I_h contributes to the resting membrane potential in Purkinje neurons (S. R. Williams, Christensen, Stuart, & Hausser, 2002). K^+-leak channels are also central to establishing the neuronal membrane potential (Goldstein, Bockenhauer, O'Kelly, & Zilberberg, 2001). The precise mechanisms that regulate resting neuronal membrane potential are poorly understood (Bean, 2007), but dystrophin may play a role in that regulation in Purkinje neurons through currently unidentified mechanisms.

Although DGC members have been linked to ion channel stabilization in the brain, much less is known about such associations in cerebellar Purkinje neurons; this is due to the apparent specificity of the DGC and interconnectedness with constituent proteins in these neurons and the lack of research investigating such relationships in these

neurons. Syntrophin, a cytoplasmic member of the DGC, has been implicated in clustering various ion channels and transporter proteins that are central to regulating neuronal excitability. Syntrophin interacts directly with dystrophin at its C-terminal (as reviewed in Blake et al., 2002). In membrane extracts from whole-brain tissue, syntrophin forms a complex with Na^+ channels (Gee et al., 1998), but this has not been examined specifically in the cerebellum. Syntrophin has been shown to interact with inward rectifying K^+ channels in the cerebellum, as illustrated using protein-purification techniques (Leonoudakis et al., 2004). Syntrophin proteins also have a cell-specific pattern of localization in the cerebellum; α1- and β2 syntrophin isoforms reside in granule cells (Gorecki et al., 1997), whereas γ1- and γ2 syntrophin isoforms are found in Purkinje neurons (Alessi et al., 2006). The Purkinje neuron-specific isoforms, however, do not appear to form the strong association with dystrophin that is found with α- and β-isoforms (Alessi et al., 2006). Although whole-brain extracts reveal an enrichment of syntrophin in postsynaptic fractions, this is not the case in the cerebellum. In the cerebellum, syntrophin is largely located in the endoplasmic reticulum of Purkinje neurons, quite distinctly different from the localization of dystrophin in these neurons (Alessi et al., 2006). As expected from their distinct localization patterns, syntrophin and dystrophin proteins do not form a strong interaction in Purkinje neurons (Alessi et al., 2006), nor is syntrophin down-regulated in the *mdx*^{3vc} mouse strain that lacks both short and long isoforms of dystrophin (Moukhles & Carbonetto, 2001). In muscle, the lack of dystrophin results in disruption of much of the DGC in both DMD (Ervasti et al., 1990) and the *mdx* model (Ohlendieck & Campbell, 1991). These data imply that impaired recruitment of Na^+ and/or K^+ channels to the postsynaptic membrane would not be

expected to be perturbed in the *mdx* mice in a manner similar to that seen in muscle and in other brain regions, principally through associations with syntrophin, and therefore suggest that the neuronal DGC is much more heterogeneous than its counterpart in muscle and much less understood.

Unlike syntrophin, the cytosolic DGC member, β-dystrobrevin exhibits extensive colocalization and interaction with dystrophin in Purkinje neurons. This is noted from observations of dystrobrevin knock-out transgenic mice and in the spontaneous mutant, *mdx* mice, as the loss of one cytoskeletal protein results in corresponding loss of the other (Grady et al., 2006). In brain extracts, β-dystrobrevin binds to a more recently identified DGC member, dysbindin (Benson et al., 2001). Dysbindin is present within Purkinje neuron somata, but unlike dystrophin and β-dystrobrevin, it does not extend into the dendritic arbor. Rather, dysbindin distribution is limited to the primary apical dendritic shaft (Sillitoe et al., 2003). Dysbindin is also present in mossy fibre synaptic glomeruli (Benson et al., 2001; Sillitoe et al., 2003). The localization of dysbindin is upregulated specifically in lobule IX of the cerebellar vermis in *mdx* mice, with no corresponding upregulation in the lateral hemispheres (Sillitoe et al., 2003). This is surprising, given the reported loss of the dysbindin binding partner, β-dystrobrevin, in the *mdx* cerebellum. Whether this upregulation occurred specifically in Purkinje neurons was not detailed in the study by Sillitoe and colleagues. These findings reveal that particular areas of the cerebellum are more affected than others by the absence of dystrophin, as found in the current study. Little is known about the role of dysbindin in the CNS or the functional consequences of its upregulation in the *mdx* mouse cerebellum. A better understanding of the exact constituents of the DGC in Purkinje neurons, the degree of interconnectedness

amongst them, and the consequences of their absence and/or disruption throughout the cerebellum awaits further investigation.

An interesting hypothesis proposed to explain pathological processes in both muscle and brain in the dystrophic organism is Ca^{++} dysregulation. In dystrophic muscle tissue, the loss of dystrophin and its associated DGC leads to increased intracellular Ca^{++} levels that contribute to its pathology (Wrogemann & Pena, 1976; see Whitehead, Yeung, & Allen, 2006 for review). In the *mdx* mouse sensorimotor cortex, the number of neurons positive for the cytosolic Ca^{++}-buffer, calbindin, is increased relative to controls (Carretta et al., 2003). In the *mdx* cerebellum, Ca^{++}-channel kinetics are aberrant in granule cells, as channels remain open substantially longer than in WTs (Haws & Lansman, 1991). Basal Ca^{++} levels are thus elevated in granule cells (Hopf & Steinhardt, 1992). Moreover, *mdx* mice display altered levels of cerebellar LTD (J. L. Anderson et al., 2004; 2010). Since LTD is a cellular correlate of learning that is Ca^{++}-dependent (Sakurai, 1990), it will be important to investigate the potential for region-specific disruption of Ca^{++}-channel kinetics in Purkinje neurons in the cerebellum.

Interestingly, knockout of the PMCA2 calcium transporter in mice results in elevated intracellular Ca^{++} levels in Purkinje neurons (via reduction in Ca^{++} efflux). That knockout also alters intrinsic Purkinje neuron membrane properties, resulting in hyperpolarization of the membrane potential and a decrease in frequency and regularity of spontaneous firing (Empson et al., 2010). Results reported for the lateral region Purkinje neurons from *mdx* mice in the present study are consistent with the work by Empson and colleagues. This is intriguing, given that dystrophin and its complex have been implicated in Ca^{++} transporter stabilization in heart tissue (J. C. Williams et al.,

2006), although this relationship has not been studied extensively in neuronal tissue. As with the reported associations with Na^+ and K^+ channels, the documented association between DGC proteins and the Ca^{++} transporter occurs via binding to syntrophin, levels of which are unaffected in mice lacking dystrophin. Although there is sufficient evidence to suggest Ca^{++} disruption in the *mdx* mouse brain, basal Ca^{++} levels have not been examined in *mdx* Purkinje neurons, nor has the ability of these neurons to buffer Ca^{++} via mechanisms, such as calbindin regulation. The extent of calcium dysregulation, if any, in *mdx* Purkinje neurons is unknown.

3.4.3. Implications

This is the first report of aberrations in Purkinje neuron function in the lateral cerebellum, the region relevant to cognition, in the *mdx* mouse. The dramatic hypoexcitability demonstrated in Purkinje neurons lacking dystrophin in this model reveals a possible explanation for the noted cognitive deficits observed in DMD patients. As Purkinje neurons are the only source of output from the cerebellar cortex in both vermal and lateral cerebellum, dysregulation of their intrinsic membrane properties would be expected to severely compromise cerebellar function.

Ataxia is a common symptom of cerebellar dysfunction (Schmahmann, 2004) and has been attributed to aberrations in the intrinsic membrane properties of Purkinje neurons as a consequence of ion channel dysregulation (Walter et al., 2006). Perturbations in intrinsic firing properties in *mdx* mouse Purkinje neurons would therefore be expected to produce ataxic symptoms. Ataxia, however, is not a widespread finding in studies of *mdx* mice (Vaillend et al., 1995), although mild ataxia (possibly

recorded due to muscle weakness) was reported in an early study of older *mdx* mice (Bulfield et al., 1984). Studies report impairments in motor function, affecting balance (Grady et al., 2006), righting reflex, and negative geotaxis in *mdx* mice (Rafael et al., 2000). However, the effects of dystrophin deficiency on balance and coordination seem relatively mild in their phenotypic presentation in *mdx* mice, since signs are not obvious without specific behavioural testing. Although this mild motor phenotype is difficult to document in animals with progressive muscle weakness, the observed phenotype is consistent with the reduced distribution of dystrophin in the vermal relative to the lateral region in WT mice (Snow et al., unpublished, reported in Chapter 2) and the smaller number of parameters affected in vermal relative to lateral Purkinje neurons in *mdx* mice in the present electrophysiological study.

The dramatic reduction in endogenous firing in *mdx* mouse Purkinje neurons in the lateral cerebellum would be expected to compromise the functional output of this region, and may be considered to contribute to a "mental ataxia". Hyperpolarization of Purkinje neurons and reduced excitation would be expected to reduce the overall inhibitory influence of the cerebellar cortex on the deep cerebellar nuclei and their subsequent targets. This change would disrupt information transfer along cerebrocerebellar loops that are thought to be the basis of the cerebellar contribution to mental processing. In fact, in PMCA2-deficient mice, the Purkinje neurons exhibit a similar electrophysiological phenotype of membrane hyperpolarization, decreased spontaneous firing frequency and regularity (Empson et al., 2010) to that reported here in the lateral cerebellar region in *mdx* mice. Those mice exhibit impaired cerebellar function, including severe ataxia (Kozel et al., 1998) and impaired motor performance on

behavioural tests (Empson, Turner, Nagaraja, Beesley, & Knopfel, 2010). Cognitive abilities, however, have not been evaluated in these mice. In *leaner* mice affected by P-type Ca^{++}-channel mutations, Ca^{++} current density and firing regularity are decreased (Walter et al., 2006), as is performance on both motor and cognitive tasks (Alonso et al., 2008). In addition to firing irregularity, *mdx* mice exhibit alterations in multiple parameters affecting spontaneous and evoked firing that may contribute to noted learning impairments in these mice (Muntoni et al., 1991;; Vaillend et al., 1995; 2004). The functional consequences of dystrophin deficiency on cerebellar-associated learning and mental processing, however, are unclear. Future research using established testing paradigms, for example, the conditioned eye-blink response (for review, see Villarreal & Steinmetz, 2005), would determine the degree to which cerebellar-mediated learning is disrupted and advance our understanding of mental processing deficits in the *mdx* mouse.

3.4.4. Conclusions

The DGC and associated dystrophin have been implicated in stabilizing the multiple ion channels that are central to intrinsic neuronal excitability. The results of this study provide additional support for this role by revealing a significant decrease in the excitability of Purkinje neurons in dystrophin-deficient *mdx* mice that coincided with a significant hyperpolarization of the membrane potential. As intrinsic membrane properties are determined by the interplay of various ionic conductances, the results of these current clamp experiments merit further investigations of particular ion channel function in voltage-clamp experiments as well as ion channel localization using

immunohistochemical studies as a function of cerebellar region and in the absence of dystrophin.

The findings of this experiment highlight the importance of distinguishing cerebellar functional zones in future work characterizing Purkinje neuron electrophysiology, especially as it relates to cognition, including investigations into dystrophin deficiency and cognitive deficits in DMD. Results support the hypothesis that cognitive deficits associated with DMD could, indeed, be mediated by the loss of dystrophin protein, particularly in the lateral cerebellum. Results also reveal a putative mechanism by which cognitive deficits in DMD may arise, through dysregulation of intrinsic membrane properties of Purkinje neurons, the principal cells of this structure.

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Chapter 4

General Discussion

4.1. Overview of Findings

The central aim of my research was to investigate the distribution and role of dystrophin in cerebellar Purkinje neurons. Employing multiple techniques (immunostaining of sections and patch clamping of isolated Purkinje neurons), the thesis project was designed to study dystrophin-deficient *mdx* and wild-type mouse cerebellum as a means of revealing potential neurophysiological causes of cognitive impairments associated with DMD. Several lines of evidence point to cerebellar dysfunction in brains lacking dystrophin and correspondingly suggest putative mechanisms by which cognitive deficits in DMD could arise. A key deficiency in this literature, however, is the lack of information on Purkinje neuron function when dystrophin is absent, specifically in the lateral cerebellar region, the key functional zone that subserves the cerebellar modulation of non-motor, mental skills.

The first experiment aimed to determine the precise localization of dystrophin, both between functional zones and within specific subcellular locations. A detailed immunohistochemical and morphometric analysis revealed a homogeneous pattern of localization of dystrophin in the mouse cerebellum in both vermal and lateral cerebellar regions. This is important, given the heterogeneous distribution noted in other brain regions, such as the cerebral cortex. Moreover, this experiment highlighted that dystrophin is present throughout the Purkinje neuron dendritic arbor. The localization observed in this thesis work is in contrast to the distribution of other DGC constituents found in Purkinje neurons, notably the restricted distribution of dysbindin, which is

confined to the main apical branch of Purkinje neurons (Sillitoe et al., 2003). Comparisons of the subcellular distribution of dystrophin versus other DGC proteins are relevant for understanding their interactions with each other, and, hence their roles in Purkinje neuron function.

The ubiquitous presence of dystrophin in Purkinje neurons throughout the cerebellum strongly suggests that this protein plays a key role in typical Purkinje neuron function. Semi-quantitative analysis on 192 Purkinje neurons demonstrated that dystrophin is preferentially distributed in the lateral cerebellum relative to the vermal region. The general notion in the literature is that an increased presence of this protein in the cerebellum suggests that dystrophin-dependent functions will be markedly impaired in its absence. If the same logic is applied when considering the regionalization of functions within the cerebellum, these findings suggest that functions subserved by the lateral region will be more severely affected by the absence of dystrophin from Purkinje cells in the lateral hemispheres. From the cellular level, this is exactly the result of the study of Purkinje neuron function. Although significant functional perturbations were found in *mdx* mouse Purkinje neurons regardless of region, including membrane hyperpolarization and irregular firing, the differences in the frequency of spontaneous activity only reached statistical significance in the lateral region.

The large number of regional differences noted in both spontaneous and evoked activity in dissociated Purkinje neurons was surprising. In fact, more significant differences were noted (e.g., by statistical analysis) according to cerebellar region than dystrophin status (genotype, i.e., wild-type vs. *mdx* mice). The results of the immunohistochemical study demonstrate that the level of dystrophin varies between

regions (higher in the lateral region), and the results of the electrophysiological experiment demonstrate corresponding functional differences in Purkinje neurons. Although the precise level of dystrophin was not quantified in isolated Purkinje neurons used in the electrophysiological study, future correlation of that information to electrophysiological findings would provide even more convincing evidence of the involvement of dystrophin in intrinsic membrane properties of Purkinje neurons in WT mice. The results demonstrate an effect of dystrophin on membrane potential and excitability, since in WT mice, a relative membrane depolarization and increased firing frequency were found in Purkinje neurons from the region with the greater density of dystrophin (the lateral cerebellum). As well, the main finding from between-genotypes comparison was a hyperpolarization of the membrane potential and a corresponding decrease in firing frequency and regularity in mice lacking dystrophin. These frequency differences were maintained when Purkinje neurons were stepped to similar membrane potentials. This latter finding suggests that Purkinje neuron excitability is generally diminished when dystrophin is absent (in *mdx* mice) or diminished (vermal cerebellum).

Collectively, these data further implicate the absence of dystrophin in cerebellar dysfunction, as is consistently reported in the literature, albeit without studying the lateral region of the cerebellum that contributes to non-motor, mental processing. These experiments show, for the first time, that a lack of dystrophin perturbs intrinsic Purkinje neuron function. Moreover, this is the first study focused on functional differences between vermal and lateral cerebellar Purkinje neurons and also the first report of altered cerebellar function in the lateral region based on loss of dystrophin. Therefore, this body of work provides strong support for theories postulating that generalized cognitive

dysfunction in DMD can be attributed to dysregulation of cerebrocerebellar circuitry as it demonstrated that one of the central classes of neurons in that loop, Purkinje neurons, are perturbed.

4.2. Relevance to Previous Research: Immunohistochemical Studies and Cytoplasmic Dystrophin

In the immunostaining experiments, double labeling with dystrophin and calbindin was used to identify Purkinje neurons. Calbindin delineates Purkinje neuron morphology as it fills the axon, the soma, and the dendrites, and therefore allows precise localization of dystrophin. This is particularly clear for the Purkinje neuron somata, when observations can be made using z-stacks to focus through the entire soma. Analysis of the compiled images across the focal plane demonstrated that dystrophin localized to the somatic membranes of Purkinje neurons, with no detectable immunoreactivity in the cytoplasm; this is consistent with the majority of reports on dystrophin localization in these neurons (Knuesel et al., 1999; Lidov et al., 1990). As previously mentioned, one group of researchers noted high levels of dystrophin diffusely throughout the soma (Huard & Tremblay, 1992; Huard et al., 1992). A possible explanation for this inconsistency may relate to differences in the detection methods used by that laboratory compared to the present experiment and others. Diffuse cytoplasmic labelling of dystrophin was noted by Huard's lab when biotinylated secondary antibodies were used to detect the primary anti-dystrophin antibody. Notably, the same anti-dystrophin antibody was used in the present research and the previous reports by Huard and colleagues. The authors (Huard et al., 1992) speculated that the sensitivity of detection

using the avidin-biotin method was higher than for immunofluorescent methods reported previous to their studies (Lidov et al., 1990). It is worth noting as well that Purkinje neurons possess high levels of endogenous biotin, particularly within the soma (McKay, Molineux, & Turner, 2004), a feature of Purkinje neurons that was not known at the time of the studies by Huard and colleagues. Immune-detection artifacts likely underlie the discrepancies in the literature regarding dystrophin localization within Purkinje neurons, but direct comparison of the two methods under controlled conditions in a single experiment would be required to confirm this speculation.

4.3. Relevance of Findings to Other Theories of Cognitive Deficits in DMD and *mdx* mice: nitric oxide (NO)

Although the lack of brain dystrophin is presumed to cause CNS impairments in DMD, the precise physiological mechanisms behind this etiology are still largely unknown. Blake and Kroger (2000) proposed that a lack of nitric oxide (NO), secondary to the loss of dystrophin, may be a principal contributor to brain abnormalities and cognitive deficits associated with DMD. NO is a gaseous signalling molecule involved in many cellular signalling pathways within multiple tissues (Bredt, 1996). Arguments for the possible relevance of NO to brain deficits associated with a lack of dystrophin come from studies of NO and dystrophin in muscle. An enzyme responsible for NO production, the neuronal isoform of nitric oxide synthase (nNOS), is localized to the sarcolemma by the dystroglycan complex (DGC) in muscle through its association with syntrophin (Brenman et al., 1996). The severe downregulation of the DGC, including the nNOS-binding partner syntrophin, in both DMD and *mdx* mice results in a lack of sarcolemmna-

localized nNOS, and its product, NO (Brenman, Chao, Xia, Aldape, & Bredt, 1995). In the *mdx* mouse model, nNOS is ectopically localized to the cytoplasm rather than the sarcolemma (Brenman et al., 1995). Genetically addressing the NO-deficiency in dystrophic mice with transgenic overexpression of nNOS significantly attenuates the dystrophic phenotype (Wehling, Spencer, & Tidball, 2001). Increasing the level of NO through pharmacologic treatment of *mdx* mice also alleviates the progression of dystrophy and improves muscle regeneration (Mizunoya, Upadhaya, Burczynski, Wang, & Anderson, 2011). In both these interventions to the *mdx* mouse, restoring NO levels is beneficial despite the persistent lack of dystrophin from muscle.

Although there is sufficient evidence demonstrating NO dysregulation in muscle when dystrophin is absent and the importance of NO regulation in ameliorating muscle pathology, the evidence to argue for a NO-mediated effect for the physiological findings documented in the present study is weak. There is evidence that syntrophin and nNOS associate in the brain, as shown in crude extracts from rat brain (Hashida-Okumura et al., 1999), consistent with the reported association in muscle. Both syntrophin (Alessi et al., 2006) and nNOS (Egberongbe et al., 1994) are present in cerebellar Purkinje neurons. Involvement of a lack of NO in the pathophysiology affecting Purkinje neurons in the *mdx* mouse cerebellum could be speculated based on the observation that cerebellar LTD, which is impaired in the *mdx* brain, is NO-dependent (H. Ogasawara, Doi, & Kawato, 2008). In contrast to the case in muscle, however, nNOS levels are unaffected in the *mdx* mouse brain (Deng, Glanzman, & Tidball, 2009), including the cerebellum (Sillitoe et al., 2003; J.E. Anderson, unpublished data), thereby precluding such an interpretation.

There are several lines of research to account for preserved levels of nNOS in the *mdx* brain. As previously mentioned, the specific γ-syntrophin found in Purkinje neurons does not form a strong association with dystrophin (Alessi et al., 2006), which would account for preservation of syntrophin in the dystrophic model that lacks all the dystrophin isoforms, *mdx*^{3cv} mice (Moukhles & Carbonetto, 2001). In turn, preservation of the nNOS binding partner, syntrophin, would be expected to maintain intact nNOS levels. Additional evidence would argue for preserved nNOS levels in Purkinje neurons in the absence of dystrophin, as the binding domain of the Purkinje-specific γ-isoform possesses a PDZ domain distinct from that seen in the nNOS- binding domain in α- and β-isoforms in muscle (Alessi et al., 2006). Moreover, although a syntrophin-nNOS interaction was demonstrated in brain tissue (Hashida-Okumura et al., 1999), it was found with α1-syntrophin, not present in Purkinje neurons (Alessi et al., 2006). Collectively, these findings argue against a NO-mediated effect for the physiological findings of the present study. The presence of alternative binding regions in CNS-located syntrophins suggests syntrophins associate with as-yet-unidentified ligands within Purkinje neurons. Although a lack of brain dystrophin in the *mdx*^{3cv} mouse, including long and short isoforms, does not affect the levels of brain syntrophin (Moukhles & Carbonetto, 2001), γ-syntrophin regulation has not been examined specifically in the *mdx* mouse cerebellum, or in comparisons of vermal and lateral cerebellar Purkinje cells in wild-type brain. It should not be assumed, however, that the consequences of two distinctive deficiencies, lack of full-length dystrophin vs. lack of all dystrophin isoforms, will be the same. Given the specificity of the γ-syntrophin isoform found in cerebellar Purkinje neurons and its unique binding properties relative to other syntrophin isoforms, future studies of the

relationship of γ-syntrophin and dystrophin in vermal vs. lateral Purkinje neurons in the cerebellum in wild-type and *mdx* mice are justified.

4.4. Developmental Differences or Differences in Mature Neurons?

Several studies have reported similar differences in action potential characteristics across age, in which there is a developmental shift from broad, low-amplitude spikes to narrow, large-amplitude spikes (Fry, 2006; Hockberger, Tseng, & Connor, 1989; McKay & Turner, 2005). In dissociated Purkinje neurons from mice, however, these properties reach maturity by P12, after which time they are indistinguishable from Purkinje neurons of mice at P16-21 (Fry, 2006), the age range of mice employed in the current study. This information on Purkinje neuron development argues against a simple developmental interpretation of the findings from the present study.

In a developmental study of intrinsic membrane properties of isolated Purkinje neuron somata in mice, bursting was the more immature phenotype, with tonic firing patterns appearing later in development (Fry, 2006). An increase in bursting behaviour in vermal regions overall in both WT and dystrophic mice, and in the *mdx* lateral cerebellum, could be thought to reflect a developmental lag in vermal relative to lateral Purkinje neurons and in those lacking dystrophin. However, there are several lines of evidence that argue against this interpretation. Firstly, firing frequency in dissociated Purkinje neurons from mice reaches mature, stable levels by P12, days prior to the youngest mice used in the present study. Secondly, current understanding of the complexities of Purkinje neuron development is not compatible with an argument for developmental differences as the causative factor of effects due to dystrophin deficiency.

For example, Purkinje neuron birthdates vary by approximately two embryonic days, with both early- and late-born Purkinje neurons present in both vermal and lateral functional regions (Voogd, 2012). As well, Purkinje neuron maturation is very complex and does not proceed homogenously by lobule, since lobules that are considered late-developing (VI, VIII) still contain areas in which Purkinje neurons develop earlier relative to others in the same zones (Goodlett, Hamre, & West, 1990). This developmental specificity extends beyond lobules, as Purkinje neuron development varies even amongst neighboring Purkinje neurons in a given lobule (Sotelo & Dusart, 2009). Thirdly, given the six-day postnatal age window from which neurons were sampled, developmental effects would be expected to be negligible. Lastly, the mean age of animals did not differ between region ($p = 0.6$) or genotype ($p = 0.4$). Therefore, it seems very unlikely that the differences found in the present study between cerebellar functional regions could be explained by developmental differences between regions, and are therefore interpreted to reflect true regional differences in electrophysiologically mature Purkinje neurons.

4.5. Appropriateness of the *mdx* Model to Investigate DMD-related Cognitive Deficits

The *dystrophin* gene is very large, and its expression is regulated by a complex system that produces numerous heterogeneously-localized proteins isoforms, including full-length dystrophins in muscle and central neurons as well as truncated Dp71, Dp116, Dp140, and Dp260 isoforms (Perronnet & Vaillend, 2010). Given the considerable variability in cognitive dysfunction associated with DMD (Cotton et al., 2001; Wingeier

et al., 2011), research has sought to identify particular mutations within the dystrophin gene that are associated with cognitive impairment. Mutation site has been correlated with the degree of cognitive impairment in DMD. Mutations in the more distal part of the gene sequence affect the shorter isoforms of dystrophin and are associated with a more severe cognitive phenotype than that seen with mutations predominantly affecting full-length brain isoforms (Taylor et al., 2010). Specifically, mutations affecting Dp140 (Felisari et al., 2000; Moizard et al., 1998; Taylor et al., 2010; Wingeier et al., 2011) and Dp71 (Moizard et al., 1998; 2000), both present in glial cells (Pilgram et al., 2010) are associated with more severe cognitive dysfunction in DMD, whereas gene mutations in the promoter region encoding brain-type full-length dystrophin are compatible with a typical IQ range (den Dunnen et al., 1991; Rapaport et al., 1992).

Research to date, therefore, suggests that deficiencies of the truncated dystrophin isoforms in the brain have a more deleterious effect on cognitive abilities than an absence of full-length neuronal dystrophin. However, specific cognitive impairments, namely limited immediate verbal memory across all intellectual levels (Hinton et al., 2000) are reported in DMD. This is coupled with the consistent finding that IQ scores are shifted downward by one standard deviation in DMD relative to the general population (S. Cotton et al., 2001). Together, these features suggest that there is a primary neural deficit in DMD even in the absence of severe cognitive dysfunction and mental retardation that are typically associated with mutations affecting truncated dystrophin.

The precise functions of the various brain isoforms of dystrophin are not completely understood. However, recent research using mutant mice lacking short-form Dp71 (Daoud et al., 2008), full-length dystrophin in the *mdx* model (J. L. Anderson et al.,

2003; J. L. Anderson et al., 2004), and mice in which all dystrophin brain isoforms are drastically reduced, the *mdx*^{3cv} model (Vaillend et al., 1998), has identified important roles for this class of proteins in synaptic function and learning.

There are obvious differences between *mdx* mice and DMD, as *mdx* mice show a milder phenotype, including longer relative life span, less severe muscle pathology, and less severe motor deficits than seen in the human condition (as reviewed in Banks & Chamberlain, 2008). As well, the genetic contribution in *mdx* mice is restricted to a single mutation site compared to the genetic heterogeneity of DMD (Bulfield et al., 1984). Although the variability in genetic determinants of DMD and the range of cognitive phenotypes as a result precludes the development or use of a unifying model for investigating cognitive dysfunction associated with the disorder, it is argued that these differences in *mdx* mice and DMD are precisely the advantage to using the *mdx* model as a tool to investigate *specific* cognitive deficits associated with a lack of dystrophin in an organism, and therefore make this model superior over other mouse models.

Firstly, the fact that *mdx* mice lack only the full-length isoform of dystrophin makes them a more elegant model to examine the consequences of neuronal functioning in the absence of a single form of dystrophin. This rationale is especially compelling given the important and diverse localization patterns of the different dystrophin isoforms in the brain. Moreover, *mdx* mice lacking only the full-length isoform of brain dystrophin do display specific memory and learning deficits, as described above. The *mdx* mouse model is likely more reflective of the bulk of those with DMD, as the majority of those afflicted fall within the normal range of IQ, typically associated with mutations affecting brain full-length dystrophin alone (den Dunnen et al., 1991; Rapaport et al., 1992) versus

mutations affecting multiple isoforms that are associated with more severe cognitive deficits (Felisari et al., 2000; Moizard et al., 1998; Taylor et al., 2010; Wingeier et al., 2011).

A second major advantage to investigating the neuronal effects of a lack of dystrophin using the *mdx* mouse relate to the milder musculoskeletal phenotype of these mice compared to those with DMD. As noted, *mdx* mice display less severe forms of muscle pathology and consequential motor deficits than seen in DMD. Although this feature may be a disadvantage in some regards as a means of investigating the resultant muscle pathology and potential treatments in DMD (Banks & Chamberlain, 2008), it confers an advantage for investigating neuronal function and associated cognitive deficits associated with a loss of brain dystrophin. This is due to the reduced burden on researchers to tease apart the behavioural consequences of dystrophin deficiency in muscle from those in brain (Sekiguchi, 2005). Significant muscle pathology and degeneration are not evident in *mdx* mice until after P21 (Pastoret & Sebille, 1995), the upper limit of age used in the present electrophysiological study. As such, any potential influence of muscle-related pathology and consequential motor deficits on Purkinje neuron electrophysiology was minimized by using the *mdx* mouse model of DMD for this thesis project.

Despite these advantages to the model system used in the present study, there are limitations. As previously noted, in addition to its localization in Purkinje neurons, dystrophin is also typically present in pyramidal neurons of the hippocampus and cerebral cortex (Lidov et al., 1990). Both brain regions are implicated in working memory in both humans (Karlsgodt, Shirinyan, van Erp, Cohen, & Cannon, 2005) and rodents (Yoon,

Okada, Jung, & Kim, 2008). As such, functional deficits may arise in these regions as a consequence of an absence of neuronal dystrophin and may, therefore, play a role in the reported memory and learning deficits in those with DMD and in *mdx* mice lacking brain dystrophin. Studies examining hippocampal function in *mdx* mice demonstrate alterations in long-term potentiation (Vaillend, Billard, & Laroche, 2004), a proposed cellular phenomenon thought to underlie learning and memory (Bliss & Lomo, 1973). As well, spatial learning, traditionally associated with intact hippocampal function but also associated with intact cerebellar function (see Chapter 2 Discussion), is deficient in *mdx* mice (Vaillend, Billard, & Laroche, 2004). There are no detailed investigations, however, of the localization of dystrophin within specific cerebral cortical regions, including those implicated in immediate or working memory (i.e. prefrontal cortex), nor are there studies examining neuronal function in the cerebral cortex (i.e. intrinsic membrane properties, synaptic transmission or plasticity) in the *mdx* mouse. This is concerning, given the diversity of cortical functional zones, the complexity of the laminar arrangement of neurons based on functional region (i.e. sensory and motor areas differ in the depth of their input and output layers), and the molecular heterogeneity of cortical pyramidal neurons (Kolb & Whishaw, 2001), including the presence of dystrophin in only a subset of these neurons in certain cortical regions (Lidov et al., 1990).

Although perturbations in other brain regions crucial for memory may well contribute to cognitive deficits in DMD and the *mdx* model, Purkinje neuron dysfunction is not incompatible with cerebral cortical dysfunction in *mdx* mice as a means of revealing physiological mechanisms that could contribute to deficits in immediate memory, as the lateral cerebellum is intricately interconnected with cerebral cortical

regions, including the dorsolateral prefrontal cortex implicated in working memory (Strick, Dum, & Fiez, 2009). Rather, the findings of altered intrinsic membrane properties of Purkinje neurons in the present study would be expected to perturb the ability of the cerebrocerebellar network, including the dorsolateral-cerebellar pathways, to keep information "at the ready" for processing. This may occur with or without impairments in prefrontal cortical neurons that may be present in dystrophin deficiency. Again, however, there is limited data on the precise distribution of dystrophin in functional zones of the cerebral cortex or the consequences of its absence on pyramidal neuron function. Additional studies are needed to more fully understand the involvement of cortical and hippocampal regions in the memory deficits reported in those with DMD, including more detailed localization studies in the typical brain as well as electrophysiological studies of cortical pyramidal neurons lacking dystrophin. Moreover, the development of models lacking only P-type dystrophin (in Purkinje neurons) or B-type (in hippocampus and cerebral cortex) would be invaluable tools for elucidating the specific contributions of multiple brain regions in the noted cognitive deficits in cases of dystrophin deficiency.

In addition to murine models, many other animal models of DMD exist, including canine (i.e. Golden Retriever muscular dystrophy; Sharp et al., 1992), feline (Vos, van der Linde-Sipman, & Goedegebuure, 1986), and invertebrate (i.e. *C. elegans* DMD; Gieseler, Grisoni, & Segalat, 2000) models. Of these, GRMD has been extensively studied as a means of understanding the dystrophic process in muscle, as these animals exhibit a more severe muscle pathology than the milder phenotype of murine or feline models, and, thus, more closely mimic human DMD in that regard (Banks & Chamberlain, 2008; Nakamura & Takeda, 2011). Canine models, however, are more

difficult and expensive to breed and maintain relative to mouse models (Banks & Chamberlain, 2008; Nakamura & Takeda, 2011). Due, in part, to such limitations, the *mdx* mouse continues to be the most studied model to date (De Luca, 2012). This is especially true regarding studies investigating neural function and learning and memory impairments in cases of dystrophin deficiency, as there are no such studies in canine models. Interpretation of such studies, however, would be confounded by the severity of the muscle pathology and resulting motor deficits that are less severe in the *mdx* model, as previously noted, a limitation that is highly relevant when studying a brain region intimately involved in both motor and cognitive abilities, as the cerebellum.

4.6. Relevance to Purkinje Neurons *in Vivo* and Advantages of Dissociated Preparation in Electrophysiological Studies

A conspicuous feature of Purkinje neurons is their ability to fire action potentials in the absence of synaptic input. These action potentials are seen in multiple preparations of Purkinje neurons of the cerebellum, including the dissociated preparation used here. There were several advantages to using the dissociated model in the context of investigating effects of the lack of dystrophin on neuronal function. Firstly, firing properties of dissociated Purkinje neurons are remarkably similar to those in slice preparations (where the Purkinje neuron in its entirety is preserved) and to those obtained *in vivo* (Raman & Bean, 1997). The notable exception is the trimodal pattern, seen in slice preparations (McKay & Turner, 2005; Womack & Khodakhah, 2002; 2004). One of the components of this pattern, burst firing, was seen in the current study and in others using dissociated preparations (Raman & Bean, 1997; Swensen & Bean, 2003). This

consistent observation excludes the notion that dendritic conductances are *essential* for bursting behaviour. Investigating action potential properties in Purkinje neurons that have been isolated from all other sources of input and from their extracellular milieu is certainly the most direct approach to determine if a lack of dystrophin alters their endogenous membrane properties.

Secondly, the immunohistochemical study confirmed that Purkinje neuron somata are abundant in dystrophin. Given that brain dystrophin levels are highest in the cerebellum, and within the cerebellum, dystrophin levels are highest in the lateral region, particularly in Purkinje somata, the electrophysiological effects of dystrophin deficiency were examined in the single subcellular region in the CNS with the highest typical level of dystrophin present. This approach yielded the greatest chance of distinguishing a difference due to a lack of dystrophin. In fact, there were striking differences in firing frequency, particularly in lateral Purkinje somata from *mdx* vs. WT mice. In the vermal region, where dystrophin density is lower, the difference in firing frequency between *mdx* and WT mice failed to reach statistical significance. Having now established that there are deficits in endogenous activity in Purkinje neuron somata in the *mdx* mouse, it would be interesting to examine these properties in slice preparations under pharmacological blockade. Dystrophin is present along the entirety of Purkinje neuron dendrites. Based on the results of the current study, it is very likely that intrinsic membrane properties will be altered in *mdx* Purkinje neurons when these Ca^{++} conductances, abundant in dendrites, are present. Such an experiment using a slice preparation would allow for recordings to be obtained from both the soma and dendrite. This would yield a more complete picture, arguably more similar to the *in vivo* character of the effects of dystrophin deficiency in

these neurons when both the extracellular matrices and dendrites are intact. Such an experiment was originally proposed in this thesis, but technical challenges precluded completing this experiment during the course of training.

4.7. Experimental Considerations and Limitations

In electrophysiological experiments, the health of the neurons is a fundamental concern. The use of unhealthy neurons in patch clamping experiments introduces a host of problems, including difficulty with tight-seal formation, membrane depolarization artifacts, and a lack of action potential firing (Cummins, Rush, Estacion, Dib-Hajj, & Waxman, 2009). Several factors were considered in selecting the appropriate postnatal window to ensure healthy Purkinje neurons were obtained whilst ensuring validity of the results in the electrophysiological study. Brain dystrophin is found early in rodent postnatal life but increases substantially by P15 (Knuesel et al., 2000). In the WT mouse cerebellum, dystrophin is detectable at P14 (Huard & Tremblay, 1992; Huard et al., 1992). Hence, data are garnered from Purkinje neurons in which dystrophin is typically present. Although murine Purkinje neurons are not morphologically mature until P20, at which time their dendritic arbors attain maximal length (Weiss & Pysh, 1978), dissociated Purkinje neurons show "electrophysiological maturity" by P18; in fact, nearly all parameters investigated in the present study are stable by P12 (Fry, 2006). Given these prior findings, this study examined electrophysiological properties of essentially mature Purkinje somata in *mdx* and WT mice at P16-21. This age range was carefully selected to increase the ability to detect a potential difference between neurons from *mdx* and WT mice and balance this desire with the goal of maximizing neuron viability after

dissociation, which is higher in younger animals. Dissociated preparations are not ideal for examining intrinsic properties in older mice. Such studies are typically not done on Purkinje neurons from mice past P21 due to the low rate of viability following dissociation after this age. Given the results of the present study, patch clamping using cerebellar slices, where whole cell recordings can be done in older mice using pharmacological blockade to examine intrinsic membrane properties, is warranted to determine if the dystrophin-mediated changes and the noted regional differences persist with increasing age.

In muscle, dystrophin deficiency compromises the integrity of the muscle fibre membranes and renders them more susceptible to damage (Petrof, Shrager, Stedman, Kelly, & Sweeney, 1993) and, hence, to the process of muscle-fibre dissociation by enzymatic digestion (personal communication, J. Anderson). As the Purkinje neuron dissociation method also employed enzymatic digestion of proteins to liberate individual neurons, it was unclear whether *mdx* mouse Purkinje neurons would also be more fragile and more difficult to dissociate in a viable state, given their lack of dystrophin. However, this did not appear to be the case; no differences were noted between *mdx* and WT mice, in the approximate number of viable, patchable Purkinje neurons after dissociation. It could also be argued that the dissociation process affects Purkinje neurons from *mdx* mice to a larger degree than those from WT mice, due to the potential compromise to membrane integrity in the absence of dystrophin and with DGC modifications. This, however, could not explain the large number of regional differences in parameters from both spontaneous and evoked recordings. To minimize any influence of the dissociation process on all Purkinje neurons in this study, the selection of Purkinje neurons and

inclusion of data extracted from their respective recordings were carefully considered in planning the statistical analysis of this project. Firstly, data regarding spontaneous activity and membrane potentials were included only from Purkinje neurons that could withstand the protocol of evoked potentials without showing a substantial change in the amount of current required to maintain the holding potential. Such a change would indicate cellular deterioration and neuronal depolarization over time, which affects firing frequency. Secondly, recording protocols were run in the same order for all Purkinje neurons, with spontaneous activity recorded first, prior to the stimulation protocols. This pattern was utilized to minimize the inadvertent introduction of cell-to-cell variability. Lastly, although the dissociation process removes axonal and dendritic processes, some dendritic "stump" remains, and this can be very large in some dissociated Purkinje neurons. To minimize the potential introduction of cell-to-cell variability from dendritic conductances, particularly the dendritically-abundant voltage-gated Ca^{+} currents that are involved in bursting, only Purkinje neurons in which the apical stump was less than the approximate height of the soma were used for patch clamping. The finding of similar capacitance values across all groups suggests that the amount of total cellular membrane, including dendrite, did not differ based on region or genotype.

An intriguing possibility for the differences in membrane hyperpolarization noted both between regions and genotypes may be the influence of I_h, a hyperpolarized-induced cation current that is expressed in Purkinje neurons from dissociated (Raman & Bean, 1999; Swensen & Bean, 2003) and slice preparations (S. R. Williams et al., 2002). Studies of this I_h current, however, have yielded conflicting results, depending on the preparation. In dissociated Purkinje neurons, the contribution of I_h to net inward current

flowing during firing is small (Raman & Bean, 1999; Swensen & Bean, 2003), and pharmacological blockade of I_h either leaves firing frequency unaffected (Raman & Bean, 1999) or slightly increases the number of spikes in burst firing (Swensen & Bean, 2003). In somatic whole-cell recordings from slice preparations, however, pharmacological blockade of I_h results in periods of quiescence in normally tonically-firing Purkinje neurons with a concomitant large membrane hyperpolarization. This latter observation suggests that I_h serves to maintain the resting membrane potential within a dynamic range near that required to elicit the requisite Na^+-channel activation that sustains steady, tonic firing (S. R. Williams et al., 2002). Suppression of I_h current in Purkinje neurons from dystrophin-deficient mice could contribute to the hyperpolarization mediated by the absence of dystrophin that was seen in the current study, as could differences in regional distribution of the HCN1 channel through which I_h is conducted. I_h, however, is activated at very hyperpolarized potentials (greater than -80 mV), and this level of hyperpolarization is not typically seen in dissociated Purkinje neurons without stimulation (Raman & Bean, 1999). Although I_h was noted in some Purkinje neurons upon the delivery of hyperpolarized pulses, the protocol used in the present study was designed to evaluate evoked waveform properties and was not sufficient for reliable induction of significant I_h activation, which peaks in amplitude at -120 mV (Raman & Bean, 1999). These features, therefore, precluded detailed examination of the I_h current in this study.

In this series of experiments, the interpretation of differences in dystrophin distribution and electrophysiological properties was based on data gathered from neurons that were identified as belonging to functional regions according to anatomical location.

Recent evidence suggests that the functional zones of the cerebellum may not be as clearly distinguishable based on anatomical considerations as classically thought. It is commonly thought that only lateral cerebellar regions receive input from, and send projections to the cerebral cortex (Kandel et al., 2000). However, a recent study that employed retrograde labelling, documented that the vermal cerebellum also receives input from motor and premotor cortical areas in monkeys (Coffman, Dum, & Strick, 2011). The fact that the vermal cerebellum receives input from areas of the motor cortex further corroborates the involvement of this region in motor function. It is still the lateral regions that project to non-motor areas of the cerebral cortex, which initially implicated the lateral region in mediating cognition and mental functioning (Leiner et al., 1986). These new insights into cerebral-cerebellar circuitry, therefore, do not alter the interpretation of present findings, although future investigation of the impact of afferent projections on Purkinje neuron function would very usefully employ retrograde labeling techniques for more precise differentiation of Purkinje neurons based on discrete functional zones.

4.8. Further Research

The results presented in Chapters 2 and 3, in the context of previous literature, bring forth many new questions about dystrophin in the cerebellum and its relation to cerebellar function. As previously discussed, these could be addressed by:

1) conducting region-specific immunohistochemical studies of colocalizing proteins such as $GABA_A$ receptors and DGC members (i.e. β-dystrobrevin) to determine if they also exhibit regional differences in density,

2) investigating Ca^{++} regulation in *mdx* Purkinje neurons, including basal intracellular Ca^{++} concentration, the levels of associated ion-sequestering proteins such as calbindin, and ion channel localization and kinetics,

3) carrying out voltage-clamp experiments that would allow for detailed examination of ion channel function in Purkinje neurons from *mdx* mice and identification of the possible contribution of Na^{+} and K^{+} conductances to the noted perturbations in intrinsic properties, and,

4) assessing the degree to which cerebellar-mediated associative learning is affected, if at all, in the cerebellum of *mdx* mice.

Together, results of the current series of experiments and previous literature also suggest two additional avenues for future research: examination of Purkinje neuron morphology and output signalling to target projections in the DCN in *mdx* vs. wild-type mice.

4.8.1. Purkinje Neuron Morphology

Patterns of neuronal activity are consequent to an ongoing and activity-dependent refinement of synaptic connections throughout the lifespan. Dendritic branching patterns are responsive to multiple environmental experiences and perturbations and represent a key area of plastic change within the CNS (Kolb & Whishaw, 1998). The dendritic branching pattern also affects the propagation of action potentials and ion channel conductance (Vetter, Roth, & Hausser, 2001). Dendritic maturation and development have been strongly correlated with spontaneous Purkinje neuron activity (McKay & Turner, 2005; Womack & Khodakhah, 2002). As such, dendritic morphology and

branching play a key role in regulating synaptic transmission, and alterations within these processes are expected to have an impact on cellular communication.

Studies using the *mdx* mouse model have examined neuronal morphology outside of the cerebellum. Specifically, pyramidal neurons within the sensorimotor cortex of *mdx* mice exhibit increased terminal dendritic branch lengths and reduced spine density compared to wild-type controls (Minciacchi, Del Tongo, Carretta, Nosi, & Granato, 2010). The consequence of an absence of dystrophin on Purkinje-neuron morphology, however, has not been examined directly. These cerebellar neurons possess uniquely extensive dendritic arborization, the pattern of which is known to affect synaptic input-output relationships. For these reasons, it is reasonable to determine the morphological characteristics of Purkinje neurons in the *mdx* mouse brain in order to appreciate the nature and extent of cerebellar pathophysiology in the dystrophin-deficient cerebellum. This could be carried out by adding a label, such as Neurobiotin, to the patch pipette solution during electrophysiological recordings from slice preparations, followed by histological processing of tissue post-recording. Estimates, such as maximum width and length of each dendritic tree, could be obtained and used to determine the cross-sectional area of the molecular layer in which the dendritic tree is housed (as per McKay & Turner, 2005) to determine if there are regional and genotypic differences in such morphological parameters.

Functionally-relevant changes in neuronal morphology may also occur at the level of the dendritic spine, even in the absence of dystrophin. This may occur through two possible mechanisms. Firstly, the change may be a direct consequence of decreased endogenous activity by Purkinje neurons, as documented in *mdx* mice. Blockade of

spontaneous electrical activity in Purkinje neurons using TTX results in changes in both density and size of their dendritic spines (Bravin, Morando, Vercelli, Rossi, & Strata, 1999; Harvey, Morando, Rasetti, & Strata, 2005). A second mechanism through which spine morphology could be altered is through dysregulation of actin. Dystrophin contains a domain that binds to actin in both brain and muscle (Renley, Rybakova, Amann, & Ervasti, 1998). As well, actin is an integral component of the neuronal cytoskeleton and is intricately involved in the activity-dependent modulation of dendritic spines (Hotulainen & Hoogenraad, 2010). The absence of dystrophin in DMD and the *mdx* murine model of DMD may be expected to perturb the dynamic regulation of the actin cytoskeleton and, hence the formation and retraction of dendritic spines that contribute to synaptic plasticity. A recent study noted decreased spine density in distal dendrites of neocortical pyramidal neurons in *mdx* mice (Minciacchi et al., 2010). Such a phenomenon has not yet been examined in Purkinje neurons, in which synaptic plasticity is altered (J. L. Anderson et al., 2004; 2010). In fact, there are very few studies examining neuronal morphology in DMD or in the *mdx* mouse model. At present, the extent to which dystrophin disruption affects dendritic and spine morphology in Purkinje neurons is unclear. Determining the morphological characteristics of Purkinje neurons in the *mdx* mouse model is required to more fully appreciate the possible nature, extent and impact of cerebellar pathophysiology in cases of dystrophin deficiency.

4.8.2. Purkinje Neuron Output in Dystrophin Deficiency

Purkinje neurons ultimately exert influence over other brain regions through synaptic connections with the deep cerebellar nuclei. The neurons of those nuclei, in turn,

synapse onto the targeted brain regions. Although perturbations in dystrophin have been reliably shown to affect receptor clustering and impair synaptic signalling of Purkinje cells in response to synaptic input, there are no published reports on the synaptic potentials evoked by Purkinje neurons on the target deep cerebellar neurons in animal models with an altered cytoskeletal DGC. As Purkinje neuron output is inhibitory onto the DCN, hypoexcitability of Purkinje neurons in *mdx* mice would be expected to lead to a corresponding reduction in the capability for Purkinje neurons to inhibit the DCN. This would reduce the inhibition of cerebellar neuronal activity as a whole, and be in addition to the noted reduction in inhibitory influence onto Purkinje neurons as a consequence of the reduction in synaptically-located $GABA_A$ receptors. As such, multiple mechanisms appear to reduce the inhibitory influence of the cerebellum as a whole.

Impaired Purkinje-neuron output signalling has been inferred from deficits in cerebellar-mediated motor functioning in *mdx* mice, including impaired balance and deficits in the righting reflex (Grady et al., 2006; Rafael et al., 2000). Given the characteristic muscle pathology in dystrophin-deficient mice (albeit mild-to-moderate, relative to DMD) and the contribution of Purkinje neurons to motor function, investigating Purkinje neuron-DCN synaptic transmission would provide additional evidence to argue for (or against) impairment in Purkinje neuron output. As well, impaired synaptic transmission to the DCN, through which the signal processed by a Purkinje neuron ultimately exits the cerebellum, would further support the theory of dysregulation in cerebrocerebellar loops as a central feature of DMD.

4.9. Conclusions

The experiments reported in this thesis examined both the distribution of dystrophin and the electrophysiological properties of Purkinje neurons in the absence of dystrophin, taking into account the different functional zones of the cerebellum. The results provide considerable insight into the degree of cerebellar dysfunction in the absence of dystrophin and elucidate physiological mechanisms by which cognitive deficits in DMD may arise. A central finding in neuropsychological assessments in DMD is the selective deficit in immediate verbal memory. Such deficits are assumed to be at the core of CNS involvement in DMD. These deficits are further assumed to contribute to the generalized impairments in academic performance. Evidence in the literature points to the lateral cerebellum and its connectivity to non-motor areas in the cerebral cortex, as important pathways for intact immediate verbal memory capabilities. Results of electrophysiological experiments in this thesis provide further context for such a model of dysfunction by demonstrating that basic physiological mechanisms of one of the key components of this pathway, Purkinje neuron are aberrant in the absence of dystrophin. Thus, the findings from this thesis work contribute to the development of a model that attempts to explain how changes at the molecular level (dystrophin) impact functioning at the cellular level (impairments in intrinsic membrane properties in Purkinje neurons). Cellular dysfunction could be a physiological mechanism that contributes to the development of a specific deficit in cognitive processing in immediate verbal memory, now well-established in the literature.

The results of these experiments confirm a novel role for dystrophin in the CNS as a protein that is involved in regulating neuronal excitability. Importantly, present

results extend this idea from the level of the synapse to the level of the neuron in the cerebellum. Although $GABA_A$ has received considerable attention as a downstream target for the pathological impact of a lack of neuronal dystrophin, the results of several studies, including the current findings, stress the importance of probing $GABA_A$-independent aberrations in order to identify other downstream sequelae of Purkinje neurons that lack a typical protein, dystrophin.

Although such experiments are particularly relevant to studies of the neuropsychological sequelae of dystrophin deficiency, they also contribute to our understanding of the neuronal DGC. A review of the literature demonstrates that this superfamily of DGC proteins is incredibly complex and diverse, and even more heterogeneous in brain than in its muscle counterpart, likely owing to the diversity of cell types in the CNS in which the DGC is located. The present experiments reveal significant and functionally important heterogeneity between the vermal and lateral functional zones, and this heterogeneity is both at the molecular level and at the electrophysiological level. Such considerations of heterogeneity across functional zones are just beginning to come to light, despite many decades of electrophysiology experiments designed to dissect the complex functions of Purkinje neurons.

In the short term, this research fills a void in the literature on cognitive deficits in DMD by ascertaining basal electrophysiological functioning of Purkinje neurons in an established model of DMD. The experiments were necessary steps towards understanding how the absence of dystrophin affects cerebellar functioning; indeed, they were prerequisites to studies aimed at identifying specific ion channels that may be dysfunctional and/or downregulated in DMD. Collectively, this information will have

important implications for attempts to develop therapies to address cognitive deficits in DMD by extending our understanding at the molecular level, how dystrophin impacts neuronal structure and function, and should help the efforts to understand the biological basis of cognitive deficits in DMD and other conditions.

In the long term, this research has more widespread implications for understanding the role of dystrophin in cerebellar functioning and how the cerebellum contributes to non-motor learning in the intact brain. Although our understanding of cerebellar functioning and its involvement in motor behaviours has expanded immensely over the last 200 years, there remains much to be elucidated. Numerous studies have revealed a broader role for the cerebellum in overall information processing, including cognitive functioning, as initially suggested by Leiner et al. (1986). There remains much interest into how this "little brain" and its elegant, ancient and more evolutionarily recent circuitry contribute to behaviours, motor and otherwise. Dystrophin appears to be an important factor in this contribution. Lastly, characterizing the biophysical properties of neurons in the *mdx* mouse model of DMD will, in the longer term, allow researchers to determine whether potential treatments are altering not only observable, measurable behaviour but also brain structure and function. This consideration is important, as such studies are not feasible to the same degree in human studies of the disorder.

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