Roles of activity-dependent transcription factor Mef2c in the synaptic and morphological organization of early postnatal Purkinje neurons

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**NANYANG
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UNIVERSITY**

SINGAPORE

ROLES OF ACTIVITY-DEPENDENT TRANSCRIPTION
FACTOR MEF2C IN THE SYNAPTIC AND
MORPHOLOGICAL ORGANIZATION OF EARLY
POSTNATAL PURKINJE NEURONS

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Roles of activity-dependent transcription factor *Mef2c* in the synaptic and morphological organization of early postnatal Purkinje neurons

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ABBREVIATIONS

Mef2: Myocyte enhancer factor 2

TF: transcription factor

PL: Purkinje cell layer

ML: molecular layer

IGL: internal granule layer

EGL: external germinal layer

DCN: deep cerebellar nuclei

VZ: ventricular zone

RL: rhombic lip

WM: white matter

PC: Purkinje cell

GABA: gamma-Aminobutyric acid

fMRI: functional magnetic resonance imaging

ROR α : retinoic acid-related orphan receptor-alpha

GIRK2: glutamate receptor ionotropic kinase 2 precursor

ASD: autism spectrum disorder

ION: inferior olivary nucleus

CF: climbing fiber

MF: mossy fiber

PF: parallel fiber

GC: granule cell

GoC: Golgi cell

BC: basket cell

SC: stellate cell

VN: vestibular nucleus

PN: pontine nucleus

SC: spinal cord

RN: red nucleus

TH: thalamus

RetN: reticular nucleus

WT: wild-type

PCP2: Purkinje cell protein 2

shRNA: short-hairpin ribonucleic acid

miRNA: micro-ribonucleic acid

CMV: cytomegalovirus

MADS: MCM1 agamous deficiens serum response factor

HDAC: histone deacetylase

HAT: histone acetyltransferase

GFP: green fluorescence protein

GFAP: glial fibrillary acidic protein

IRES: internal ribosome entry site

mGluR2: metabotropic glutamate receptor 2

NeuN: neuronal nuclear antigen

PVA: Parvalbumin

NSPC: neural stem/progenitor cells

Bdnf: brain derived neurotrophic factor

BrdU: bromodeoxyuridine

EPSC: excitatory postsynaptic current

IPSC: inhibitory postsynaptic current

CAMKII: calmodulin kinase II

vGlut: vesicular glutamate transporter

GAD67: glutamate decarboxylase 67

NDep: not deprived

Dep: deprived

ABSTRACT

Numerous brain disorders arise from disruptions in one or more genetically-regulated processes that occur during embryonic and postnatal neuronal development. Amongst these processes, several are regulated by transcription factors but very little is understood about how they exert their functions. In this study, we first characterize the expression of the activity-dependent transcription factor myocyte enhancer factor 2c (*Mef2c*) and found that it is selectively expressed by Purkinje cells of the adult cerebellum. A spatio-temporal analysis further revealed that the onset of *Mef2c* expression occurs early postnatally which coincides with a period of dynamic synaptic and morphological changes in Purkinje cells.

In order to uncover its roles in Purkinje cell development, we knocked down *Mef2c* specifically in Purkinje cells at different stages of development. We show that a loss of *Mef2c* at P3 results in a significant increase in spine numbers of Purkinje cells at P14. A sustained increase in dendritic arborization was also observed in the Purkinje cells lacking *Mef2c* during the first three weeks of postnatal development, while other processes such as migration from P0 to P4, viability and elimination of somatic processes from P4 to P8 remain unaffected.

Furthermore, the knockdown *Mef2c* from P1 to P14 resulted in an increase in the number of GABAergic proteins in the soma and dendrites while the glutamatergic synapses remain unchanged. However, by P21, we observe a decrease in both the inhibitory synapses in the soma as well as the glutamatergic synapses on the dendrites. Because *Mef2c* has been shown to be an activity-dependent gene, we assessed the consequences of behavioral manipulation on *Mef2c* expression, and found that increase in locomotor activity from food deprivation results in an increase in *Mef2c* expression in Purkinje cells in the simple, CrusI and CrusII lobules of the cerebellum.

Collectively, our studies revealed several insights about an activity-dependent transcriptional regulator of Purkinje cell development: Firstly, we determined that *Mef2c* is expressed selectively by postnatal Purkinje cells, serving as a novel molecular marker for analysis of Purkinje cells. From the loss of function experiments, we next show that *Mef2c* is an essential regulator of dendritic arborization and spinogenesis of Purkinje cells from birth until maturity. Quantification of GABAergic and glutamatergic synaptic proteins revealed that *Mef2c* differentially controls synaptic organization at two key developmental stages of Purkinje cell development. Since both disruption in Purkinje cell function as well as the *Mef2c* gene have been implicated in neurodevelopmental disorders such as autism, our identification of the temporal and cell-specific roles of *Mef2c* during Purkinje cell development provide insight to a key molecular player implicated in pathogenesis of cerebellum-associated disorders.

1. INTRODUCTION

The thesis aims to explore the roles of activity-dependent transcription factor *Mef2c* in the Purkinje cells of the cerebellum. Although our understanding of the properties and connectivity of neuronal sub-populations has advanced over the recent years, details about the molecular mechanisms employed to carry out the proper functioning of the cerebellum, particularly GABAergic neurons, remains limited. This introduction will first describe experimentally validated and proposed involvement of the cerebellum to the overall functioning of the brain. Next, the contributions of the cerebellum to a range of motor and non-motor diseases with a focus on the inhibitory Purkinje neurons in the cerebellar cortex will be discussed. Finally, an overview of the Mef2 family of transcription factors, with a focus on Mef2c, will be covered.

1.1 An overview of the cerebellum: Function, disease and connectivity

1.1.1 A summary of cerebellar functions and related disorders

The cerebellum has traditionally been known to control motor movement and coordination (Eccles, 1967, Ito, 2006). In human case studies of total or partial cerebellar agenesis, patients were reported to show debilitating motor and cognitive deficits (Yu et al., 2014). Patients with partial or severe damage of the cerebellum also exhibit difficulties in coordination and perturbations in fine as well as coarse limb movement (Spencer et al., 2003). In addition to this, examination of patients with cerebellar-related disorders also reported irregularities in timing of movement, oculomotor reflexes and proprioception (Ivry and Keele, 1989, Weeks et al., 2017). The participation of the cerebellum in motor functions was further validated by functional magnetic resonance imaging (fMRI) analysis on human subjects as they

carried out motor-intensive tasks (Lotze et al., 1999). Although all the evidence established a strong link to motor functions, these studies did not address whether the cerebellum participated in non-motor processes of the brain.

At the same time experimental evidence for the roles of the cerebellum in motor control was accumulating, theories about its non-motor functions began to grow. One of the earliest papers published in this direction suggested that the cerebellum plays a part in the memory retention of muscular movement (Gilbert, 1974). Following this, there were several other studies carried out to suggest that the cerebellum assists other non-motor functions such as emotion, language, attention and other cognitive functions (Timmann and Daum, 2007). Although investigation of the non-motor aspects of the cerebellum continued, there was a lack of compelling evidence until fMRI studies showed the selective activation of cerebellar regions upon subjecting human patients to a series of cognitive tasks (Schmahmann, 1997). In addition to this, both direct and indirect axonal projections from the cerebellum have been mapped to many parts of the brain that are known hubs for cognitive processing (Buckner, 2013). Some of these regions include the basal ganglia, thalamus and prefrontal cortex, all three of which collectively participate in learning, emotional processing, planning and even decision making (Stoodley and Schmahmann, 2010, Stoodley et al., 2012). By this point, it was clear that the cerebellum orchestrates both motor as well as cognitive functions but the underlying molecular and cellular mechanisms remained elusive.

1.1.2 The blueprint of cerebellar synaptic organization

The cerebellum can be broadly divided into the cerebellar cortex and the deep cerebellar nuclei (DCN) (Sillitoe and Joyner, 2007, Uusisaari et al., 2007). The cerebellar cortex is structurally divided into ten distinct lobules each of which has repeating units of excitatory and

inhibitory neurons arranged in a stereotypical manner (**Figure 1-1**) (Altman and Bayer, 1978). Each of the mature cerebellar lobules can further subdivided into 3 specific layers namely the molecular layer (ML) , Purkinje cell layer (PL) and the internal granule (IGL) layer (**Figure 1-2**) (White and Sillitoe, 2013). The outermost molecular layer houses inhibitory stellate and basket neurons with a large number of basket cells aligned closely to the PL (Altman and Bayer, 1978). The IGL houses both excitatory unipolar brush and granule cells along with inhibitory Golgi and Lugaro neurons (Altman and Bayer, 1978). The PL, juxtaposed between the ML and the IGL has a neatly arranged row of inhibitory Purkinje cell somatas with their dendrites projecting into the ML (Altman and Bayer, 1978).

Figure 1-1: Anatomical topology of the cerebellum (A) The cerebellum can be medio-laterally divided into the vermis (shown in purple, pink and green) and the hemisphere (in blue) in a coronal section. (B) Sagittal section of the cerebellum has ten distinct lobules. The region in purple is the anterior part of the cerebellum. Similarly, the pink region is central, the second purple region is posterior and green is nodular. (White and Sillitoe, 2013)

Excitatory afferents are relayed to the DCN and the cerebellar cortex via two major pathways, the mossy and the climbing fibers (Altman and Bayer, 1978). The mossy fibers, arising from the pontine nuclei, spinal cord and brainstem project to the granule and Golgi cells of the internal granule layer (Ebner and Bloedel, 1984). The axons of granule cells then extend laterally into the molecular layer where they branch out to form parallel fibers (Altman and Bayer, 1978). A significant population of these fibers then terminates on the extensive dendritic arbors of the Purkinje cell that lie along parasagittal planes in the molecular layer (Ebner and Bloedel, 1984). Each Purkinje cell then integrates these excitatory signals to generate sensory as well as motor instructions to the DCN and the vestibular nuclei (De Zeeuw et al., 1994, Wassef et al., 1985). The remaining parallel fibers innervate the three interneurons of the cerebellar cortex namely stellate, basket and Golgi neurons (Ebner and Bloedel, 1984).

In addition to receiving inhibitory inputs from the stellate and basket neurons, and along with the excitatory inputs from the parallel fibers, Purkinje cells receive glutamatergic signals from the climbing fibers (Sotelo, 1975). Climbing fibers, originating from the inferior olivary nucleus (ION), initially form terminals on the soma and subsequently synapse on the primary dendrites of Purkinje cells (Desclin, 1974). This climbing fiber (CF)-Purkinje cell junction is believed to be the site where error signals, discriminating the differences between intended and executed movement, occurs in an activity-dependent manner. In both the forward and inverse models of motor control, the parallel fibers bring information about the desired movement and climbing fibers act as a guide to identify the errors in this movement. Upon receiving both these commands, Purkinje cells compute the correct signals which are subsequently sent to the effector organ (Schonewille et al., 2011). It is also noteworthy to mention that although each mature Purkinje cell receives only one CF, electrophysiological studies have shown that this

input is stronger than the cumulative excitation brought about by the PFs in the Purkinje cell dendrites (Konnerth et al., 1990). Recent studies on the specific components of the cerebellum have identified potential functions for some of these neurons but the molecular mechanisms underlying the development and organization of cerebellar neuronal subtypes are not well understood.

Figure 1-2: Neuronal connectivity in the cerebellar cortex and their targets

The figure above shows the summary of the synaptic circuitry in the cerebellar cortex. Purkinje cells represented in purple, stellate cells in blue, basket cells in pink, granule cells and parallel fibers in light green, climbing fibers in orange, Golgi cells in dark grey, deep cerebellar nuclei in

yellow, inferior olivary nucleus in brown, light grey represents the target regions from the cerebellum, dark green shows some of the inputs to the cerebellum (Altman and Bayer, 1978)

1.1.3 Mouse models and tools used for the study of the cerebellum

In order to uncover the molecular and behavioural mechanisms underlying motor and cognitive functions of the cerebellum in a replicable manner, experimentation in mice is commonly employed. One of the earliest and routinely used strategies was lesioning select neurons and regions of the cerebellum. While focal lesions carried out in the vermis of the cerebellum resulted in deficits in motor coordination, difficulties in learning, emotional behavior and eye movement, loss of tissue in the hemispheres led to more cognitive-associated problems (Bobee et al., 2000, Cole et al., 2012, Takagi et al., 2000). Additionally, optogenetic studies in mice have also been carried out to map circuits and uncover functions of specific lobules and neurons in the cerebellum (Kim et al., 2014). Therefore, with the advent of these tools, it is now possible to attain a holistic understanding of cerebellar functions and key molecular tools that contribute to these processes.

In addition to lesion and optogenetic studies, genetically modified mice have also been extensively used to characterize the functions of specific genes in proper cerebellar functioning. Some of the transgenic models used in cerebellar research includes the reeler, weaver and staggerer mice in which the expression of the genes *reelin*, *GIRK2* and *RORa* are respectively absent in the cerebellum (Mariani, 1982). Additionally, a loss of these three genes in mice independently showed overlapping deficits in motor movement and coordination in the test subjects (Kashiwabuchi et al., 1995). Furthermore, assessing a range of behavioural paradigms revealed that in addition to motor dysfunction, these mutant mice cumulatively exhibit cognitive deficits which are also hallmarks of several pathological disorders (Wang et al., 2014). Analysis

of the morphological properties of cerebellar neurons in all three of these mutant mice revealed changes in Purkinje cells in the cerebellum. The expression RORa and reelin is present in the Purkinje cells while GIRK2 expression is restricted to the granule cells (Mariani, 1982).

A common feature in several neurological disorders such as ASD and schizophrenia is the loss of Purkinje cells or disruption in Purkinje cell properties but the exact roles Purkinje cells play in these disorders remains unanswered (Ito, 2002, Rapoport et al., 2000). Could there be a critical period where Purkinje cells are particularly vulnerable to perturbations? As a major class of GABAergic neuron in the brain, do Purkinje cells regulate just the inhibitory components of synaptic transmission or could they also influence excitatory profiles in cerebellum? With Purkinje cells playing such central roles in the normal functioning of the cerebellum, it is important to uncover key variables that could contribute to cerebellum-related neurological diseases.

1.2 Development of Purkinje cells and their functional significance in the cerebellum

1.2.1 Origin and genetic profiling of Purkinje cell progenitors in the embryonic cerebellum

Purkinje cells, one of the largest neurons in the CNS, are first produced during the embryonic stages and undergo a series of developmental processes such as proliferation, migration and differentiation, until birth (Chan-Palay et al., 1981). Interspersed with the cell bodies of Bergmann glia in the PL, they serve as the primary output neurons of the cerebellar cortex (Altman, 1972). The production of Purkinje cells begins in a germinative layer known as the ventricular zone which is a transient proliferative region of the developing cerebellum (Altman and Bayer, 1978). Neurogenesis of Purkinje cells starts at embryonic day (E) 10.5 and continues till E12.5 in the ventricular zone (Wallace, 1999). From E13.5, Purkinje cells become

post-mitotic and now ascend through the sub-ventricular zone (SVZ) and the potential white matter (WM) to finally settle in the cortex (Yuasa et al., 1991). The embryonic stages of Purkinje cell differentiation begins post-mitotically at E12.5 and continues up to birth at P0 (Bertossi et al., 1986, Su et al., 2006). It is interesting to note that these neurodevelopmental processes, occurring in concert during the embryonic stages, have been shown in previous findings to be controlled by a set of regulatory genes but much is yet to be understood about their independent contributions and signaling complexes they employ to execute these functions.

Amongst many genes that regulate neurodevelopmental processes in the embryonic cerebellum, several candidates have been identified as transcription factors (TF) (Hatten and Heintz, 1995). For example, *Ptfla* has been established as an essential TF for the specification of Purkinje cells and all the other cerebellar GABAergic progenitors in the cerebellar primodium (Hoshino et al., 2005). Apart from *Ptfla*, there are other transcriptional regulators such as *Corl2* and *RORa* that are expressed specifically by Purkinje cells but at different embryonic time points and for differing durations of time (Boukhtouche et al., 2006, Minaki et al., 2008, Nakatani et al., 2014). While the functions of *Corl2* are yet to be determined, a specific deletion of *RORa* has been shown to result in the abnormal Purkinje migration and death in the developing cerebellum (Boukhtouche et al., 2006, Minaki et al., 2008, Nakatani et al., 2014). Furthermore, it has also been established in both *in vitro* and *in vivo* models that *RORa* has the potential to regulate the crucial process of dendritic arborization of developing Purkinje cells (Boukhtouche et al., 2006, Takeo et al., 2015). It is interesting to note that expression of *RORa* persists beyond the embryonic stages through adulthood and has specific parts to play at a given time point of Purkinje cell development (Takeo et al., 2015). Other transcriptional factors such as *Tfap2a*, expressed from early embryonic stages to adulthood, has been identified as a modulator of

Purkinje cell specification and is suggested to have alternate roles in adulthood (Zainolabidin et al., 2017). Detailed spatiotemporal characterization of several gene candidates over the recent years has revealed a list of Purkinje cell-specific genes which may be involved in Purkinje cell functions and their dysfunction.

1.2.2 Distinct morphological developmental periods of early postnatal Purkinje cells

Development of most neurons in the mouse brain starts during embryonic periods and persists well into the early postnatal days and even up to 2 months of age (Sotelo and Rossi, 2013). The early postnatal period also corresponds to a crucial window where many neurons, including Purkinje cells, undergo dendritic differentiation and acquire, integrate and propagate inputs from other neurons as shown in **Figure 1-3** (Gao, 2007, Sotelo and Rossi, 2013). Genetic and morphological alterations of Purkinje cells during embryonic and early postnatal stages have been linked to the manifestation of many common neurological disorders such as ASD, Rett and Angelmann syndrome (Zoghbi and Orr, 2009). Many of the aforementioned neurological disorders overlap with cerebellum-related diseases, particularly correlating to the dysfunction of Purkinje cells (Albrecht et al., 1997, Oldfors et al., 1990, Sudarov, 2013). Its high clinical relevance and along with its well-defined electrophysiological and morphological properties make Purkinje cells an ideal model for studying how neurons acquire proper morphology and analyzing how genetic manipulation might influence these importance developmental processes.

After birth, Purkinje cells undergo dynamic morphological changes within both their somatic and dendritic compartments during the first 3 weeks post birth (Sotelo and Dusart, 2009) as summarized in **Table 1-5**. At postnatal day 0 (P0), Purkinje cells are said to be in a “simple fusiform” state with long processes emerging from their spindle-like somas, also referred to as a bipolar shape (Berry M and Bradley P, 1976). From P0 to P2, Purkinje cells take up a “complex

fusiform structure during which numerous thinner and shorter dendrites emerge from the cell body while the longer primitive dendrites are shortened in parallel (Wassef et al., 1985). This phase of maturation is particularly unique as both growth and retraction occur concurrently and can prolong to P4, when the cell bodies are devoid of dendrites (Sotelo, 1985). Therefore, this period of Purkinje cell development from P2 to P4 is referred to as “Purkinje cells with regressive-atrophic dendrites” (Sotelo and Dusart, 2009).

Figure 1-3: Purkinje cell development across the first 3 postnatal weeks in the mouse cerebellum (A) Simple fusiform state of Purkinje cells at P0, (B) Purkinje cells with regressive-atrophic dendrites from P3-4, (C) Young Purkinje cells with flattened dendrites at P8, (D) Flattened Purkinje cells at P14, (E) Mature Purkinje cells at P21, (Takeo et al., 2015)

Following this phase, the Purkinje cells move on to become “stellate-shaped cells with disoriented dendrites” during which they exhibit multiple fast-extending neurites from the cell body, resembling a star (Sotelo, 1975). During this period, extending from P4 to P6, the PL becomes more defined as Purkinje cells start to align in monolayers (Sotelo and Dusart, 2009). Amongst the many neurites on the cell body, there will be about 2 to 3 thicker dendritic stems, out of which 1 of 2 of them progresses to become the primary dendrite (Sotelo and Dusart, 2009).

From P6 to P10, the primary dendrite starts to expand and form secondary and high order branchlets into the molecular layer signifying the start of the “young Purkinje cells with flattened dendritic trees” stage (Sotelo and Dusart, 2009). In the next two stages, first extending from P10 to P14 and the next from P15 to P30, majority of the changes occur in the dendritic branching patterns. In the former phase, Flattened Purkinje cells start extending their neurons laterally, attaining their maximum width by P14. Contrary to this, Purkinje cell development from P15 to P20 mainly occurs vertically with the dendrites extending both upwards and downwards to give rise to a multi-planar structure (Armengol and Sotelo, 1991, Sotelo and Dusart, 2009, Sotelo and Rossi, 2013). Purkinje cells have a genetic predisposition to acquire spontaneously occurring mutations as they continue to mature postnatally which is a period of intensive morphological and synaptic reorganization (Kapfhammer, 2004, Sajan et al., 2010). In many cases, Pukinje cells were observed to develop dendritic defects that are associated with several human neurological disorders such as ASD, fragile X and Rett syndromes (Kaufmann and Moser, 2000). Therefore, uncovering the molecular mechanisms involved in the morphogenesis of Purkinje cells will give us better insight into the underlying factors that give rise to such conditions.

1.2.3 Spinogenesis in developing Purkinje cells: A cell-autonomous event

Another process that occurs in developing Purkinje cells during the early postnatal weeks is spinogenesis which allows Purkinje cells to form synapses with other neuronal populations(Sotelo, 1975). Several cerebellar studies have been observed to alter Purkinje cell spine densities upon genetic and behavioral manipulation of the cerebellum (Lee et al., 2007, Sugawara et al., 2013, Takeo et al., 2015). Analysis of dendritic spines on Purkinje cells have shown the presence of thin, stubby, mushroom, branched and unclassified spine types, with thin and stubby spines being the most prevalent and unclassified being the least (Lee and Kim, 2005,

Lee et al., 2004). Stubby spines are well-distributed in the proximal area of the Purkinje cells which comprises of primary and secondary dendrites and is also where CFs are known to make synaptic connections (Sotelo and Arsenio-Nunes, 1976). Studies have noted that round-headed stubby spines, though less in numbers, are found in clusters whereas thin spines which possess elongated heads are thinner and more frequent than the former (Lee et al., 2004, Sotelo and Dusart, 2009). The presence of different types of spines indicate that these protrusions may play specific roles in synapse formation in the Purkinje cells. When studying spines, it is essential to corroborate anatomical data with electrophysiological readings as the sole presence of spines does not necessarily imply that they are functional (Sotelo and Dusart, 2009). This could be a strong supporting reason for why spinogenesis is suggested to be driven primarily by intrinsic contributors of Purkinje cells which occurs mostly in the first postnatal week of development.

1.2.4 Intrinsic and extrinsic properties govern postnatal morphogenesis of Purkinje cells

Formation of proper dendritic branches and synapses, spinogenesis and elimination of CF-Purkinje cell synapses as shown in **Figure 1-4** are some of the main events that take place during the first three postnatal weeks of Purkinje cell development (Armengol and Sotelo, 1991, Berry and Bradley, 1976). The dynamic changes in the different phases of Purkinje cell development are result of both intrinsic and extrinsic factors (Sotelo and Dusart, 2009). Using time-specific analysis of early postnatal Purkinje cells, studies have examined the contribution of just the intrinsic factors from the combined effects of both intrinsic and extrinsic factors (Armengol and Sotelo, 1991, Wassef et al., 1985). For example, in weaver mice, lacking granule cells and PFs, formation of dendrites in Purkinje cells showed the same patterns as WT mice until the age of P7 after which branching decreased (Berry and Bradley, 1976). Furthermore, in the absence of CF input, Purkinje cells showed reduced dendritic arborization but increased

spinogenesis (Sotelo and Arsenio-Nunes, 1976). Taken together, we can infer that extrinsic factors have critical roles during Purkinje cell development but much is left to be understood about how they interact with intrinsic determinants to regulate specific developmental processes.

Figure 1-4: Synapse elimination in the developing Purkinje cells (Kano et al., 1997)

Many genes, have been identified as intrinsic regulators of Purkinje cell development at the embryonic and postnatal stages (Sotelo and Dusart, 2009). For example, the loss of *Ptf1a* in the embryonic stages results in defects in the specification of all GABAergic neurons in the cerebellum, including Purkinje cells (Hoshino et al., 2005). The deletion of the gene *SCLIP* which is expressed robustly in Purkinje cells across development, inhibited dendritic outgrowth in the first developmental week but not in the subsequent developmental periods (Poulain et al., 2008). Similarly, transcription factor *RORa*, has been implicated in several time-dependent changes in Purkinje cell development from the embryonic stages to adulthood including migration, dendrite and spine formation and a reduction in expression of other developmentally important factors such as sonic hedgehog (Gold et al., 2003, Hamilton et al., 1996, Takeo et al., 2015). Staggerer mice lacking *RORa* specifically in Purkinje cells also display motor deficits

well into adulthood (Mariani, 1982). Therefore, these genes, along with several others have established roles in Purkinje cell development but much remains to be understood about the whether they contribute to specific processes and at which point of Purkinje cell development these influences take place.

Beyond the first postnatal week, both intrinsic and extrinsic factors have been reported to work together to regulate synapse formation and other aspects of proximal and distal branching (Sotelo and Dusart, 2009, Woodward et al., 1977). In an *in vitro* study, isolated 7-day-old Purkinje cell cultures showed less dendritic branching and more cell death than when cultured together with granule cells (Baptista et al., 1994). Furthermore, organotypic cultures of Purkinje cells with the ION have been suggested to improve the survival of Purkinje cells in vitro (Ghoumari et al., 2006). In addition to extrinsic signals from cellular neighbors, autistic and WT mice which have been subjected to chronic activity in the form of exercise, over a span of two to eight weeks, display a marked improvement in survival, motor function, dendritic branching and spinogenesis (De Bartolo et al., 2015, Huang et al., 2012, Pysh and Weiss, 1979). Collectively, these results show that external inputs in the form of PFs and CFs and chronic activities are capable of positively influencing Purkinje cell development and maintenance. Although the aforementioned reports suggest vital roles of cell-autonomous factors in Purkinje cell development, it has been shown that PFs, CFs and other extrinsic factors too have minor, noteworthy roles to play in the first postnatal week (Ghoumari et al., 2006). Therefore, it can be concluded that many questions remain unanswered about the independent contributions of intrinsic and extrinsic factors in Purkinje cell developmental regulation. Are either intrinsic or extrinsic factors more influential at a given stage? How exactly do intrinsic and extrinsic variables work with each other during development? Do intrinsic factors have differing functions

in postnatal week one versus week two? Answering these questions will help our understanding of how either or both factors regulate the many aspects of neuronal growth and maintenance in Purkinje cells and uncovered principles may operate the same in other neuronal populations in the brain.

Age	Structure	Key Features
P0		- “Simple fusiform” phase, bipolar shape - Elongated primitive dendrites which regress - Non-proliferative but still migratory - Possess supernumerary CF innervations, few MFs too
P1-P2		- “Complex fusiform” phase - Primitive dendrites shorten in length - Rigorous outgrowth of nacent neurites on soma - Both outgrowth and regression occur concurrently - PCs collaterals and DCN connectivity forms
P3-P4		- “PCs with regressive-atrophic dendrites” phase - Primitive as well as most nacent neurites go through extensive atrophy - PCs are almost devoid of neurites
P5		- “Stellate cells with disoriented dendrites” phase - Intensive outgrowth of perisomatic neurites - PCs are post-migratory and in monolayers - Shortest phase in PC development
P6-P10		- “Young PCs with flattened dendrites” phase - Polarity of PCs is decided - Definitive primary/ secondary dendrites - Initiation of tertiary and higher order branching - PFs innervate distal dendrites
P10- P14		- PCs acheive maximum width during this phase - Multiple CF terminals are pruned leaving one CF exclusively innervating the proxiamal dendrites - GABAergic innervation from stellate and basket cells are defined - More GABAergic than glutamatergic synapses on soma of PCs
P15- P20		- Dendritic branching of PCs now occurs vertically acquiring a multiplanar structure - Sharp increase in height of dendritic tree - Branching is complete by P20 - Direct innervation from MFs cease

Table 1-5: Purkinje cell developmental milestones with landmark features of each stage
(Bertossi et al., 1986, Sotelo and Dusart, 2009, Takeo et al., 2015)

1.2.5 Challenges in studying Purkinje cells: Tools used and anatomical variability

Amongst the several genetic tools that have been developed for the study of Purkinje cells, the L7 or the Purkinje cell protein 2 (PCP2) is the most commonly used mouse-derived promoter due to its ability to specifically target Purkinje cells (Oberdick et al., 1988). Since the discovery of this minimal promoter, L7 has been used in RNA silencing techniques, viral vectors and knock-in mice or a combination of all these (Sługocka et al., 2017). There are, however, some advantages and disadvantages to using the minimal L7 promoter for the study of Purkinje cells in these models. For example, knockout of a gene in mouse model would serve as a good tool for assessing the consequence on overall behavior but not necessarily for studying morphological defects as sparse labelling of cells is not achievable using this strategy. This is an important issue to be addressed as electrophysiological studies have shown that Purkinje cells send collaterals to their neighboring Purkinje cells, exerting lateral inhibition on each other, making it difficult to understand the properties of individual Purkinje cells (Valera et al., 2016, Witter et al., 2016).

Neighboring Purkinje cells have also been reported to have overlapping dendrites and also receive innervations from neurons within the same cluster of cerebellar microzones, making it almost impossible to come to a cell-autonomous conclusion about the effects of a gene knockdown (Valera et al., 2016). Therefore, particularly in the case of Purkinje cells, it is crucial to use tools that enable the sparse but complete labelling of individual cells to accurately analyze synaptic and morphological changes as a consequence of genetic and behavioural manipulations.

Different from mouse genetics, the use of viral vectors is able to by-pass some of the shortcomings of genetically modified mice models. In particular, lentiviruses are very useful tools to assess morphological changes in developmental stages as they can infect both dividing as

well as non-dividing cells stably (Washbourne and McAllister, 2002). Furthermore, they have the ability to carry larger constructs and infect neurons sparsely, which is very important for studying neuronal morphology (Washbourne and McAllister, 2002). The L7 promoter is widely used in viral vectors to transduce Purkinje cells in combination with RNA silencing constructs like short-hairpin RNAs (shRNA) (Nitta et al., 2017). The use of L7 promoter on its own was reported to show weak transduction of neurons (Manjunath et al., 2009, Nitta et al., 2017). Hence, in several studies, a stronger promoter such as cytomegaloviral (CMV) component was added onto the L7 component to improve viral efficiency (Mikuni et al., 2013). While the transcription of CMV employs RNA polymerase II, shRNAs, usually situated after the promoter, engage RNA polymerase III (Liu and Berkhout, 2011). MicroRNA (miRNA) cassettes, using specific miRNAs, facilitate both polymerases, bridge this gap (Liu and Berkhout, 2011). The use of miRNA-mediated shRNA delivery has also been reported to cause less neuronal toxicity than when used alone (McBride et al., 2008). By using these tools to effectively modify Purkinje cells, we will be able to answer the more elusive questions of Purkinje cell growth and development.

Purkinje cells are one of the well-studied neuronal populations in the brain (Sotelo and Rossi, 2013). In spite of being the subject of several key scientific breakthroughs, there are some fundamental questions that need addressing for a holistic understanding of how Purkinje cells work. As it has been mentioned previously, analysis of Purkinje cells in different Zebrin⁺ and Zebrin⁻ zones showed that they differ in electrophysiological properties, highlighting the importance of categorizing Purkinje cells accordingly (Albergaria and Carey, 2014). Comparing findings to an earlier report which revealed that Purkinje cell loss was significantly higher in lobules that were late maturing versus lobules that were early maturing (**Figure 1-6, A**) (Altman,

1969, Goodlett et al., 1990, West et al., 1990). Furthermore, Purkinje cells in early versus late maturing lobules have different morphologies (McKay and Turner, 2005) (**Figure 1-6, D**).

Figure 1-6: Spatio-temporal variability of Purkinje cells in the cerebellum

(A) Early and late maturing lobules shown in green and blue respectively. (B) Distribution of apical and basal Purkinje cells in a cerebellar lobule. (C) Traced apical and basal Purkinje cells showing variability in morphology. (D) Traced Purkinje cells taken from different lobules of the cerebellum at P12 showing non-uniform maturation. (McKay and Turner, 2005)

Purkinje cell morphologies have also been reported to vary within the same lobule (Cerminara et al., 2015). Apical Purkinje cells are located along the gyrus and straight banks of the lobules while basal Purkinje cells can be found in the sulci (**Figure 1-6, B**). Apical Purkinje

cells have one primary dendrite and two secondary dendrites and are not as wide as the basal Purkinje cells which have two primaries and four secondary dendrites (**Figure 1-6, C**) (Ślugočka et al., 2017, Sotelo and Rossi, 2013). Thus whether the functions of basal and apical Purkinje cells differ from each other, remains a potential avenue to be explored. Purkinje cells have also been reported to show different sensitivities to the enriched environment paradigm across the vermal and hemispheric regions found along medio-lateral axis of the cerebellum (De Bartolo et al., 2015, Gelfo et al., 2016). Although it is unknown of how and why these Purkinje cells behave and appear different morphologically, the above studies show that it is necessary to define the region, lobules and even the type of Purkinje cell to study, especially during development.

1.3 *Mef2* family of transcription factors and their functions in the brain

1.3.1 Mef2 homologues: structure, activation and expression in the CNS

There are four known homologues of the *Mef2* genes: *Mef2a*, *Mef2b*, *Mef2c* and *Mef2d* but there is limited knowledge available on their respective roles in the cerebellum (Lin et al., 1996, Lyons et al., 1995, Shalizi and Bonni, 2005). Expression analysis in the developing mouse brain revealed that although *Mef2* is detected at E8.5 in the neural crest, it is not until E12.5 that its expression is robust and continues to be expressed into adulthood in cortical neurons (Lyons et al., 1995). This developmental expression profile coincides with the onset of key processes such as migration, specification and differentiation of immature neurons, suggesting that *Mef2* may contribute to these functions in the developing brain (Edmondson et al., 1994). An *in situ* hybridization study of the *Mef2* transcripts in the CNS showed that *Mef2a*, *Mef2c* and *Mef2d* are expressed in the neurons of cerebral cortex, hippocampus, olfactory bulb and the cerebellum while overall *Mef2b* levels in all brain regions were negligible (Lyons et al., 1995). Although the

RNA expression of *Mef2* in the CNS has been determined, little remains known about the precise identity of the neuronal subtypes expressing these genes and their functions in the brain.

1.3.2 Mef2a and Mef2d and their potential functions in the brain

In spite of knowing the region-specific distribution of *Mef2* in the brain, the details of how they exert their activities are not well understood. Are the four gene homologues expressed in distinct brain regions and neuronal subpopulations? What are the functions of the individual *Mef2* TFs in different neuronal subtypes? Many TFs are known to regulate diverse developmental programs in neurons but little is known on the precise downstream molecules and mechanisms that coordinate these processes. A genome wide association study was conducted in an effort to molecularly profile the genetic program that comes into play upon *Mef2* activation (Flavell et al., 2006). Activation of *Mef2* was achieved by adding KCl into cultured primary neurons *in vitro* while behavioural paradigms such as novel exploration was used to do the same *in vivo* (Flavell et al., 2006). Analysis using ChIP, qPCR and mRNA microarrays revealed a wide range of gene targets in both the synaptic and nuclear compartments (Flavell et al., 2006). Examples of some of these genes are *Arc*, *Bdnf*, *Ube3a* and *Nos1ap* many of which had previously been noted for their participation in synaptic and neuromodulatory tasks and have been implicated in several neurological disorders (Flavell et al., 2008).

Mef2 TFs have been identified as downstream targets of Calcineurin and p38-MAPK both of which are activated by extracellular calcium suggesting that they regulate many neuronal processes in an activity-dependent manner (**Figure 2-7**) (McKinsey et al., 2002, Shalizi and Bonni, 2005). Entry of calcium through Ca^{2+} ion channels, which increases intracellular calcium and activates neurons is essential for neuronal maturation and survival (Gallin and Greenberg, 1995). Collective evidence from these studies suggested that *Mef2* TFs, which are

tightly regulated by calcium, may contribute to neuronal viability by altering the activation levels of neurons (McKinsey et al., 2002). A study carried out in cultured cerebellar granule cells showed that *Mef2a* modulates neuronal survival with the help of calcineurin in a calcium-dependent manner (Mao and Wiedmann, 1999). Prior to this it was reported that *Mef2a* has the ability to dimerize and commonly forms heterodimers with *Mef2d* (Shalizi and Bonni, 2005). Subsequent *in vitro* studies further implicated both *Mef2a* and *Mef2d* in neuronal survival (Gaudillière et al., 2002, Li et al., 2001).

However, *Mef2a* and *Mef2d* knockdown studies using RNAi showed many effects in addition to neuronal survival (Shalizi and Bonni, 2005). Noticeable reduction in the excitatory synapses and spines, together with malformed dendritic claws were some of the consequences of *Mef2a-Mef2d* double knockdown in cultured hippocampal and cerebellar granule neurons and striatal neurons of the NAc (Flavell et al., 2006, Pulipparacharuvil et al., 2008, Shalizi and Bonni, 2005). Additionally, *Mef2*- mediated transcription of a cluster of brain-specific miRNAs was reported to modulate dendritogenesis of cultured rat hippocampal neurons (Fiore et al., 2009). Findings from these studies suggest that *Mef2a* and *Mef2d* play diverse neuronal specific roles in not just the survival but the overall development of the brain by controlling synapse formation and dendritogenesis of neurons *in vitro*. However, it is important to note that in all the aforementioned studies, the analysis was carried out *in vitro*, which may not accurately recapitulate *in vivo* conditions.

Figure 1-7: A summary of the activity-dependent processes regulated by *Mef2* transcription factors (Lin et al., 1996, Shalizi et al., 2006)

In order to further confirm the functions derived from *in vitro* studies mice with selective deletion of *Mef2c* were generated using a variety of promoters (Potthoff and Olson, 2007). A mouse with hGFAP-Cre mediated deletion of *Mef2a* surprisingly did not show anatomical or behavioural deficits (Akhtar et al., 2012). Since previous reports have suggested that *Mef2a* usually works as heterodimers with *Mef2d*, a second mouse lacking both gene homologues was generated (Akhtar et al., 2012). Apart from motor dysfunction and enhanced hippocampal short-term plasticity, no other inadequacies were reported (Akhtar et al., 2012). Finally, a triple knockout mouse, with an additional *Mef2c* deletion resulted in increased apoptosis, reduction in overall brain size and early postnatal lethality suggesting indispensable roles for *Mef2c* during brain development (Akhtar et al., 2012, Flavell et al., 2008). Contrary to these results, triple

inducible knockouts of *Mef2a*, *c* and *d* in adult neural stem/progenitor cells (NSPurkinje cell) *in vivo*, resulted in increased survival of neurons (Latchney et al., 2015). Also while some studies have shown that *Mef2* is a positive modulator of spinogenesis (Cole et al., 2012, Shalizi et al., 2006), many have reported otherwise (Flavell et al., 2006, Pulipparacharuvil et al., 2008). Collectively, data accumulated from studies provide strong evidence that *Mef2* TFs modulate spinogenesis in several parts of the brain but little is known about how it is affected in the cerebellum, particularly in the inhibitory neurons.

1.3.3 Mef2c: An overview of expression pattern in the developing cerebellum

Mef2c is the first member of the *Mef2* TFs to be detected in the CNS (Leifer et al., 1993). *In situ* hybridization studies at different developmental stages show the presence of *Mef2c* at E17 in the cortical plate but not in the ventricular zone in mice (Mao et al., 1999). At that point in development, the mouse cortical plate consists mostly of differentiating cells while the ventricular zone mostly houses actively dividing neural precursors (Molyneaux et al., 2007). BrdU assays reveal, that *Mef2c* is expressed predominantly by differentiating, postmitotic neurons in the late embryonic stages of brain development suggesting its roles in differentiation, migration and maturation of neurons (Mao and Wiedmann, 1999). Although *Mef2c* is first detected in the embryonic stages, expression does not peak until the day of birth (Leifer et al., 1994). Expression of *Mef2c* in the adult brain showed high levels of *Mef2* transcripts in the hippocampus, frontal cortex, amygdala and entorhinal cortex (Leifer et al., 1994). *Mef2c* was also found scattered in some neuronal populations of the thalamus and the cerebellum (Leifer et al., 1994, Leifer et al., 1993). Unlike many other developmentally critical TFs, once it sets in, the expression of *Mef2c* is maintained through adulthood, suggesting that it plays roles in some postnatal processes but much of its contributions remain unexplored.

1.3.4 A review of the known and potential roles of *Mef2c* in different neuronal subtypes

One of the more well-defined roles of activity-dependent *Mef2* TFs is their ability to inhibit the onset of apoptosis in response to changes in calcium-dependent signaling cascades (Heidenreich and Linseman, 2004). Similar to the other members of the *Mef2* TF, *Mef2c* is involved in neuronal survival (Heidenreich and Linseman, 2004). Purkinje cell12 cells, when subjected to oxidative stress, upregulates the production of Erk5 which is a known regulator of survival and an upstream modulator of *Mef2c* (Suzaki et al., 2002). Upon inhibiting Erk5, there was a sharp reduction in *Mef2c* levels which also coincides with an increase in apoptosis(Suzaki et al., 2002). On the other hand, control conditions without Erk5 inhibitors showed elevated levels of both Erk5 and *Mef2c*, suggesting that they interact with each other to carry out anti-apoptotic functions (Suzaki et al., 2002). A similar study showed that a calcium-dependent increase of *Mef2* transcripts in cerebellar granule neurons also exhibited higher survival than the control which lacked *Mef2c* (Mao et al., 1999). Contrary to these findings, conditional *in vivo* knockout of *Mef2a*, *Mef2c* and *Mef2d* in nestin-driven NSPurkinje cell did not display any changes in proliferation or survival (Latchney et al., 2015). These results therefore suggest that *Mef2c* might have crucial roles to play in neuronal survival and that it is essential to validate the viability of the neurons before carrying out further molecular and functional analysis.

Neurons lacking *Mef2c* exhibit a reduction in mEPSC in the adult CA1 region of the hippocampus (Li et al., 2008). Granule cells of the dentate gyrus in mice with in hGFAP-Cre or CaMKII-Cre mediated deletion of *Mef2c* have increased mEPSCs and spine numbers (Adachi et al., 2016, Barbosa et al., 2008). With these contradictory findings, it has not been determined whether *Mef2c* is a positive or negative regulator of excitatory synapses. Cre- mediated viral knockdown of *Mef2c* in a subset of layer 2/3 cortical neurons of P1 pups showed that mature

cortical neurons lacking *Mef2c* have decreased local excitatory input and an increase in excitation involving long-range circuits highlighting a dual role for *Mef2c* in this region (Rajkovich et al., 2017). A more comprehensive study of *Mef2c* conditional deletion in the cortical excitatory neurons also resulted in a significant increase in inhibition, parallel to a notable decrease of excitation (Harrington et al., 2016).

Cumulatively, these studies suggest that *Mef2c* plays important roles in many neurodevelopmental processes such as differentiation, maturation, spinogenesis and excitatory synapse formation. However, all the studies carried out so far have been in excitatory neurons, restricted to the cerebral cortex with a limited analysis of neuronal morphology. What does *Mef2c* do in inhibitory neurons? *Mef2c* exerts effects on spinogenesis but does play a role in dendrite formation? Expression of *Mef2c* has been documented in the cerebellum but a cell-type specific analysis has not yet been carried out. Hosting several inhibitory and excitatory microcircuits, the cerebellum makes an ideal brain region for the study of roles of *Mef2c* in neuronal developmental processes.

1.3.5 Clinical implications of Mef2c dysfunction with a focus on the haploinsufficiency syndrome, an emerging neurodevelopmental disorder

Several behavioral deficits that have been associated with *Mef2c* dysfunction. Mutant mice with selective deletions of *Mef2c* have been reported to display deficits in learning and memory due to defects in synapse formation (Barbosa et al., 2008, Cole et al., 2012). The partial loss of *Mef2c* has also been commonly associated with an increased susceptibility to schizophrenia (Mitchell et al., 2017). Mental retardation, abnormal movements, epilepsy and a range of ASD-like features have been linked to *Mef2c* dysfunction (Paciorkowski et al., 2013). Genetic profiling revealed that all subjects had a partial deletion of *Mef2c* which overlapped with

5q14 mutations in some cases (Novara et al., 2010, Zweier and Rauch, 2011). Recent studies conducted on a mouse model of *Mef2c* haploinsufficiency revealed that one of the possible contributors of these neurological disorders is an imbalance in levels of excitation-inhibition in the brain (Tu et al., 2017). Although progress is being made in this direction, there is currently very limited understanding of how the disruption of *Mef2c* expression in the cerebellum may cause these phenotypes.

In summary, dysregulation of *Mef2c* in the brain seems to have adverse effects on anatomical, electrophysiological and behavioural properties. A majority of the *Mef2c* related disorders appear to have origins in the early stages of neuronal development and maturation. Most of the behavioural data obtained from studying genetic manipulations suggests that *Mef2c* contributes more to cognitive than motor functions. In spite of this, expression analysis has reported the presence of *Mef2c* RNA in the Purkinje cell of the cerebellar cortex which is primarily known for motor coordination in both human and mouse systems. Purkinje cells residing in the Purkinje layer of the cerebellar cortex have been implicated in several cognition-related disorders and show overlapping symptoms as those seen with *Mef2c* deletions. Hence the question arises; where is *Mef2c* found in the cerebellum and what are its possible functions?

2. HYPOTHESIS AND AIMS

2.1 Hypothesis

We hypothesize that:

- The expression of *Mef2c* is restricted to Purkinje cells in the cerebellar cortex
- *Mef2c* coordinates changes in dendrite and spine formation during the development of Purkinje cells
- Synaptic reorganization in developing Purkinje cells is modulated by *Mef2c*

2.2 Aims

2.2.1 Determine the expression pattern of members of the Mef2 transcription factor in the cerebellar cortex

Previous studies characterizing the expression of *Mef2* family of transcription factors in the brain reported the presence of all four transcripts in the cerebellum (Lyons et al., 1995), but the exact regions have not been clearly defined. A microarray analysis (B.Barres and N.Heintz, unpublished) generated using the RiboTag system and cerebellar neuronal subtype-specific Cre mouse lines, verified that out of the four family members, *Mef2a*, *Mef2c* and *Mef2d* have robust distribution in cerebellar neurons. Expression analysis of all four *Mef2* transcripts will be carried out to validate the results from the microarray and to determine whether they are expressed in overlapping or exclusive subsets of cerebellar neuronal populations.

2.2.2 Characterize Mef2c expression using known cerebellar neuronal markers

Our microarray data (B.Barres and N.Heintz, unpublished) and previous *in situ* hybridization analysis suggest that, unlike *Mef2a* and *Mef2d*, high levels of *Mef2c* transcripts are

exclusively expressed by the Purkinje cells of the cerebellum (Lyons et al., 1995). To validate if the RNA expression coincides with the protein distribution, colocalization analysis with known protein markers of cerebellar neurons will be performed to confirm if *Mef2c* protein, like the RNA, is found exclusively in Purkinje cells.

2.2.3 Define the spatio-temporal expression of Mef2c across the developing cerebellar Purkinje cells

Many genes have distinct expression patterns and levels throughout the developing cerebellum. For example, *Corl2* is expressed robustly in the embryonic cerebellar Purkinje cells but its expression diminishes in the early postnatal and adult stages (Minaki et al., 2008). This kind of variation in expression suggests that genes may have specific roles at different time points of cerebellar development. In order to determine if *Mef2c* could play specific roles during cerebellar development, the spatio-temporal expression of *Mef2c* will be analyzed at key time points.

2.2.4 Determine the expression of Mef2c in Zebrin-defined zones of the cerebellum

The cerebellum has been reported to have microzones which can be identified by either the presence or the absence of zebrin (Hawkes and Herrup, 1995). Expressed only by the Purkinje cells, electrophysiological studies of Purkinje cells in Zebrin⁺ and Zebrin⁻ zones have reported that they have different electrical profiles with the former having less spontaneous activity than the latter (Albergaria and Carey, 2014). Microarray data suggests that *Mef2c* is also present in Purkinje cells, but the distribution in specific microzones has not been defined. Therefore, with the use of immunohistochemistry, we set out to characterize the distribution of *Mef2c* in microzones of the cerebellum, delineated by Zebrin.

2.2.5 Morphological analysis of genetically manipulated Purkinje cells

Purkinje cells are known to undergo dynamic changes in morphology during the early postnatal days of development but the molecular mechanisms that govern these changes are not well understood (Sotelo and Dusart, 2009). For example, the knockdown of ROR α at different developmental stages results in disruptions of specific processes within this window of time (Takeo et al., 2015). Using lentiviral vectors, we will explore the possible roles of *Mef2c* in controlling several aspects of Purkinje cell morphological development such as spinogenesis and dendrite formation.

2.2.6 Assess consequences of the loss of Mef2c on synaptic profile of Purkinje cells

Proper balance between inhibitory and excitatory inputs regulates important function of Purkinje cells such as LTP and LTD, which in turn play crucial roles to shape motor learning (Schonewille et al., 2011). Furthermore, an imbalance in the excitation to inhibition ratio has been highlighted as the underlying cause of several neurological phenotypes such as seizures, loss of memory and social dysfunction (Power et al., 2011, Rubenstein and Merzenich, 2003, Yizhar et al., 2011). However, there is very little information available about how changes in synaptic profiles, particularly in dynamic stages of Purkinje cell development, might be altered upon genetic modification of Purkinje cells. We therefore set out to determine if excitatory and inhibitory puncta distribution is altered upon genetic manipulation of Purkinje cells, and to what extent each of these inputs is affected through this process.

2.2.6 Explore consequences of behavioral manipulation on Mef2c expression in Purkinje cells

Mef2c, along with several other immediate early genes (IEG) such as *Arc* and *Npas4*, modulates neuronal functions like memory formation, in an activity- dependent manner

(Bramham et al., 2010, Sun and Lin, 2016). Similarly, studies have reported the roles of *Mef2c* in neuronal survival and memory (Barbosa et al., 2008) in other regions of the brain, but little is known its roles in the cerebellum. We first sought to check the levels of *Mef2c* upon increased locomotor activity and analyze its expression in the cerebellum in a region-specific manner.

3. MATERIALS AND METHODS

3.1 Animals and ethics statement

All experiments conducted were approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the guidelines stated by the National Advisory Committee for Laboratory Animal Research. The mice used for this study were of the C57BL/6N strain. Animals were housed in both the Biological Research Center under the management of the Agency for Science, Technology and Research (A*STAR) and Animal Research Facility, LKC school of medicine, Nanyang Technological University, Singapore. Animals were provided *ad libitum* access to food and water and were reared on a 12-hour light-dark cycle.

3.2 Data acquisition

All experiments were conducted in at least 2 independent rounds, with most experiments carried out 3-4 times. All numbers including animals used, puncta, dendrites, spines and cells counted are stated in each figure legend. Outliers were earmarked using the in-built function in GraphPad Prism.

3.3 Histology

Harvesting the brain

For *in situ* hybridization, mice were euthanized using a CO₂ chamber and incubated overnight in 4% Paraformaldehyde. On the subsequent day, fixed brains were transferred into 30% sucrose solution and dehydrated overnight following which they were mounted in Optimal Cutting Temperature [Sajura Finetek #4583] compound and frozen using dry ice.

For immunohistochemistry (IHC), mice were anaesthetized by injecting 2.5% Avertin intraperitoneally. (0.025g/mL pf 2, 2,2-Tribromoethanol [Sigma #T48402] mixed with 2-Methyl-2-butanol [Sigma #152463]). Once they were completely sedated, cardiac perfusion was carried out first with 0.9% saline, followed by 4% paraformaldehyde (PFA) [Sigma #P6148] in 0.1M phosphate buffer [$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ from 1st base #1542 mixed with $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1st base #1540, diluted in MilliQ water]. The brains were then transferred to 30% sucrose in MilliQ water [1st base #1091] and allowed to dehydrate overnight. Once they had sunk in the sucrose bed, they were mounted in Optimal Cutting Temperature [Sajura Finetek #4583] compound and quickly frozen in dry ice.

Sectioning the brain

Brains used for all the *in situ* hybridization analysis, characterization of *Mef2c* expression and food deprivation were sectioned on a cryostat machine [Fisher Scientific #12-5550-15] in 20 μm thickness. Sections were collected on Superfrost plus microscopic slides and [Thermo Fisher #4951PLUS4] were allowed to dry in room temperature for 20 minutes. Sections were either used immediately or stored in -80°C .

All tissues used for the morphological analysis of Purkinje cells were 100 μm thick and sectioned using a vibratome [Leica VT 1000 S]. Sections were either used directly for immunohistochemical analysis or stored in tissue collection solution (TCS) [25% Glycerin, 30% Ethylene Glycol, 1.38g/L monosodium phosphate and 5.48g/L disodium phosphate] at -20°C until further use.

3.4 In situ hybridization

RNA probes with approximate lengths of 900bps were generated by following the subsequent steps. **Table 3-5** shows the sequence designs for the forward and reverse primers used to make the RNA probes for *Mef2a*, *Mef2b*, *Mef2c*, *Mef2d* and *GAD67*.

The T7 polymerase sequence 5'-GCGCTAATACGACTCACTATAGGG-3' was added to the 5' end of the reverse primer. Total cerebellar RNA of mouse origin [Clontech #636660] was reversed transcribed [Thermo Scientific #K1652] to generate template cDNA. A classical polymerase chain reaction [i-DNA Technology #iTaq-2] was carried out with the primers and the resulting product was labeled with either 11-Digoxigenin (DIG)-UTP [Roche #11175025910] or 12-Fluorescein-UTP [Roche #11685619910].

Gene	Probe sequence
<i>GAD67</i>	5'-CCATCTCGCAAGCAACTACA-3'
	5'-AATGCACAGTGTGGGTTTCA-3'
<i>Mef2a</i>	5'-CAGCCAGCTCAACATTAGCA-3'
	5'-ACCCATGTGTCCATCCTCAT-3'
<i>Mef2b</i>	5'-GTGCTTTGTGACTGCGACAT- 3'
	5'-GAACTCTGTGGAGGCTGGAG - 3'
<i>Mef2c</i>	5'-TGATCAGCAGGCAAAGATTG-3'
	5'-ATGCGCTTGACTGAAGGACT-3'
<i>Mef2d</i>	5'-CACTCCTTCCCTGGTGACAT- 3'
	5'-CGGTCTCATAGGATCCTCCA - 3'

Table 3-5: Sequences of the probes used to characterize the expression of *Mef2* transcripts using *in situ* hybridization.

Tissue sections were fixed for 10 minutes in freshly prepared ice-cold 4% PFA [Sigma #P6148]. The slides were then washed thrice with phosphate buffer saline (PBS) [1st base #BUF-2040-10X4L] following which they undergo a 10 minute digestion with 1µg/mL of Proteinase K [Promega #V3021]. The tissues were they re-fixed in 4% PFA for 5 minutes and washed again in PBS. The sections were then acetylated for 10 minutes in a solution containing triethanolamine [Sigma #108-24-7] and acetic anhydride [Sigma #411000] dissolved in MilliQ water.

Hybridization solution was prepared with the following reagents: 50% formamide [Promega #H5052], 5X SSC [1st base #BUF-3-50], 5X Denhardts solution [Ficoll type 400 Sigma #F9378], polyvinylpyrrolidone [Sigma #PVP40] and bovine serum albumin [Sigma #A7906], 200µg/mL yeast RNA [Sigma #R6625-25G] and 500µg/mL herring sperm DNA [Promega #D1816]. The samples were prehybridized with this hybridization solution for 2 hours at 63°C. In the incubation time, RNA probes were diluted in hybridization solution with varying concentrations from 400 to 1500 ng/mL and heated to 80 °C for 10 minutes. Post prehybridization, the slides are drained and hybridization solution containing probes (either only DIG or DIG mixed with Fluorescence) are carefully loaded onto the respective samples and incubated overnight in humidified conditions at 63°C. 5X [1st base #BUF-3-50] and 0.2X SSC [1st base #BUF-3-50] buffers were made and preheated in a water bath set at 63°C. The slides were first submerged into 5X SSC where the coverslips were removed. Following these two washes with 0.2X SSC buffer was carried out for 30 minutes each. The tissues were then washed one more time in 0.2X SSC at room temperature for 5 minutes before being transferred to TN buffer [1st base 0.1M Tris-HCL pH7.5 mixed with 1st base #BUF-1416 pH7.5 and 1st base #BUF-1112 0.15M NaCl].

For chromogenic in-situ, samples which were hybridized with DIG labeled probes were then incubated with a blocking buffer made with 10% sheep serum [Sigma #SS2263] in TN buffer for

2 hours at room temperature. After blocking, the excess buffers is drained of the slides and were incubated overnight in anti-DIG- Alkaline Phosphatase antibody, diluted 1:2000 in blocking buffer. On the subsequent day, samples were washed with TN buffer and conditioned using a equilibration buffer made from 100mM Tris-HCL pH9.0, 150mM NaCl and 1mM MgCl for 5 minutes before being incubated with BCIP/NBT substrate [Promega #SS3771] for varying amounts of time. Upon sufficient colour development, the enzymatic reaction is stopped by washing with ice-cold methanol and mounted using Dako mounting medium [Dako #CS703].

3.5 Construction of shRNA plasmids

A pLL3.7 [Addgene #11795] construct with the following cassette was used for cloning the shRNA sequences: U6-shRNAMef2 (A/C)-CMV-GFP. shRNA for *Mef2c* were designed using the Invitrogen BLOCK-iT RNAi Designer, with restriction sites for HpaI and XhoI and a hairpin sequence of 5'-TTCAAGAGA-3' as shown in **Table 3-6**. DNA oligos for the shRNAs were purchased from Integrated DNA technologies, Inc. DNA and tested for the best knockdown efficiency. Sense and antisense oligonucleotides were annealed at 100°C for 8 minutes and gradually cooled down to room temperature. After digesting pLL3.7 with XhoI [New England Biolabs #R0146S] and HpaI [New England Biolabs #R0105S], the cut vector is purified using gel extraction [Qiagen #28706]. The digested pLL3.7 is then ligated with the shRNA for *Mef2c* with the T4 DNA ligase enzyme [New England Biolabs #M0202T] and incubated at 16°C overnight for 20-22 hours. The ligase is then deactivated by heating to 65° for 10 minutes. To obtained bacterial colonies with the above constructs, the ligation mix is transformed into ECOS X competent cells [Yeastern #FYE610-10VL] and incubated in a 37°C overnight. Single colonies were then picked and cultured in Luria broth containing 100µg/mL Ampicillin overnight at 37°C overnight [Sigma #A9518-25G]. The construct is then lysed from the cells by

carrying out mini-prep [Invitrogen #K210011] and digested with XbaI [New England Biolabs #R0145S] and NotI [New England Biolabs #R3189S]. The product is subsequently sequenced to assess the accuracy of the oligonucleotides and then used for further experiments.

Design and generation of overexpression constructs for Mef2c

A pCDH lentiviral vector [Systems Biosciences #CD531A-2] with the following sequence was used: EF1-MCS-IRES-RFP (EF1-Elongation factor1, MCS-multiple cloning site, IRES- internal ribosome entry site, RFP- red fluorescent protein). pUC 57 vectors containing the open reading frame sequence with kanamycin resistance was purchased from GeneArt [Invitrogen]. Both the pCDH and the pUC57 vectors were digested with XbaI [New England Biolabs #R0145S] and NotI [New England Biolabs #R3189S] and ligated overnight at 16°C for 20-22 hours with T4 DNA ligase enzyme [New England Biolabs #M0202T]. Following this, purification and transformation is carried out in the same way as mentioned above for pLL3.7.

Gene	shRNA sequence (5'>3')
shMef2c	TCGAGAAAAAAGCTTGCACTAGCACTCATTTATCTCTTGAATAAAT GAGTGCTAGTGCAAGCA
	TGGTCTCCTGTTGACAGCTTGATTCAAGAGATCAAGCTGTCAACAG GAGACCTTTTTTC
Scrambled Mef2c	TGCTCAGCCAAAGACGTGGTAATTCAAGAGATTACCACGTCTTTGG CTGAGCTTTTTTC
	TCGAGAAAAAAGCTCAGCCAAAGACGTGGTAATCTCTTGAATTACC ACGTCTTTGGCTGAGCA

Table 3-6: Nucleotide sequences of primers used to make shRNAs for knockdown studies

pCDH lentiviral vector [Systems Biosciences #CD531A-2] with the following sequence was used: EF1-MCS-IRES-RFP (EF1-Elongation factor1, MCS-multiple cloning site, IRES-internal ribosome entry site, RFP- red fluorescent protein). pUC57 vectors containing the open reading frame sequence with kanamycin resistance was purchased from GeneArt [Invitrogen]. Both the pCDH and the pUC57 vectors were digested with XbaI [New England Biolabs #R0145S] and NotI [New England Biolabs #R3189S] and ligated overnight at 16°C for 20-22 hours with T4 DNA ligase enzyme [New England Biolabs #M0202T]. Following this, purification and transformation is carried out in the same way as mentioned above for pLL3.7.

3.6 Transfection and Western Blotting

HEK293T cells maintained in Dulbecco's Minimum Essential Media (DMEM) containing [Science Werks #L0104-500] 10% fetal bovine serum [Gibco #10082147] and 5% Pencillin-streptomycin [Gibco #15140122] were seeded into 6-well plates and incubated overnight to allow them to adhere. Plasmids of 1µg concentration were transfected into each of the wells using OPTI-MEM [Gibco #31985070] and Lipofectamine 2000. All the above reagents were mixed and incubated for 20 minutes at room temperature before introducing it to the cells. After incubation for 48 hours, proteins were extracted using M-PER reagent [Pierce #78501] mixed with the protease inhibitor cocktail. Quantification was then carried out using calorimetric assay reagent [Bio-Rad #500-0006]. 40µg of the protein samples was subsequently used to carry out SDS-PAGE on a 10% acrylamide gel. Once the proteins run out, transfer was carried out using the iBlot Dry Blotting System [Invitrogen] and the membranes were blocked in 5% Bovine Serum Albumin [Sigma #A9306] for 2 hours at room temperature. Primary antibodies were diluted in blocking solution and allowed to incubate overnight. Dilutions are as follow: 1:500 anti-*Mef2c* [Santa cruz #sc-13266], 1:1000 Gapdh [Sigma #G8975]. The following day the

membrane is washed with TBS-T (10mM Tris, 150mM NaCl, 0.5% Tween 200 and incubated with the corresponding secondary antibodies conjugated to horse radish peroxidase in 1:3000 dilutions. The blots were developed using chemiluminesce reagents [Cell signaling #688P3] and captured on x-ray films [Thermo Fisher #34090].

3.7 Lentivirus production

Post testing in HEK cells, viral particles with titers $>10^8$ TU/mL were commercially produced by VectorBuilder (Cyagen Biosciences) according to the construct designs provided. The L7 plasmid (Sawada et al., 2010) was a gift from Dr Hirokazu Hirai, Gunma University, Japan.

3.8 Intracranial injection of lentiviral vectors into neonatal mice

Intracranial injection of lentiviruses was carried out on 1, 3 and 7-day-old pups as described in (Adesnik et al., 2008) with some differences. The pups were separated from their mothers and anesthetized on ice until immobilized. This process is done one pup at a time to avoid the basal body temperatures from dropping drastically. L7-shRNA-IRES-GFP lentivirus is first diluted to 10^6 by mixing with 2.5mg/mL Fast Green FCF dye [Sigma] which was used to help in visualizing the site and spread of the virus injected. An anesthetized pup was then placed on an ice pack and its head is wiped down with a cotton swab soaked in 70% ethanol. 2 μ L of virus was introduced into each injection site along the late maturing lobules of the cerebellum. Bilateral injections are carried out on lobules 4-8 with one injection on each lobule. As the skin of young mice pups is translucent, the lambda suture point is visible and acts as a landmark for the injection along the medio-lateral and rostro-caudal axis of the cerebellum. The needle is inserted perpendicular to the skull at a depth of 500 μ m which is then held in place for 3

minutes to prevent backflow. Since injections were carried out in the same litter, sterile tattoo ink was injected into the palms to distinguish the control from the experimental pups. Subsequently, all the injected pups are placed on a heating pad till they recover and are returned to their home cage where they are held until the specific experimental time-points.

3.9 Immunohistochemistry (IHC)

For 20 μ m sections on slide

Tissues were first permeablized for 10 minutes with 0.2% Triton-X [OmniPur #9400] diluted in Phosphate Buffer Saline [First Base]. The slides were then blocked with 3% Horse Serum [Invitrogen #16050122] in 0.1% Triton-X [OmniPur #9400] in PBS. Primary antibodies were diluted in blocking buffer according to the **Table 3-10** below and added onto the slides before incubating them overnight at 4°C. Corresponding secondary antibodies diluted 1:1000 each. Alexa fluor 488 [Molecular Probes #A-21206], Alexa fluor 555 [Molecular Probes #A-21431] and Alexa flour 647 [Molecular Probes #A-31571]. Freshly prepared 0.1 μ g/mL DAPI [Sigma #D9542] was prepared and the slides were incubated for 10 minutes and mounted with Prolong Gold [Molecular probes #P36930]. Mounting agent was allowed to set overnight away from light before subsequent confocal imaging.

For 100 μ m sections

Free-floating IHC was carried out for 100 μ m sections. Brain tissues sectioned using the vibratome were either stored in TCS solution [25% Glycerin, 30% Ethylene Glycol, 1.38g/L monosodium phosphate and 5.48g/L disodium phosphate] as mentioned previously or used freshly cut for this process. The sectioned were first washed with Phosphate Buffer Saline [First Base] and subsequently permeablized using 0.3% Triton-X [OmniPur #9400] diluted in PBS for

1 hour at room temperature. After washing out the Triton-X solution with PBS, the tissues were incubated for another hour in blocking solution composed of 3% Horse Serum [Invitrogen #16050122] in 0.1% Triton-X [OmniPur #9400] in PBS. Sections were immunostained for 2 nights in appropriate concentrations of the respective antibodies. Subsequently, tissues were stained with secondary antibodies Alexa fluor 488/555/647 [Molecular Probes #A-21206], and 0.1µg/mL DAPI [Sigma #D9542] and mounted using Prolong Gold [Molecular probes #P36930].

S.No	Antibody	Dilution	Company
1	Rabbit anti- <i>Mef2c</i>	1:500	Cell signaling
2	Mouse anti-mGluR2	1:1500	Advanced Targeting systems
3	Mouse anti-NeuN	1:1000	Millipore
4	Mouse anti- Calbindin	1:5000	Swant
5	Goat anti-Parvalbumin	1:2500	Swant
6	Rat anti-GFP	1:1000	Nacalai Tesque
7	Mouse anti-GAD67	1:1000	Millipore
8	Ginea pig anti-vGluT2	1:2000	Millipore
9	Rabbit anti-GFAP	1:500	Sigma Aldrich
10	Ginea pig anti-Zebrin	1:1000	Frontier institute

Table 3-10: List of antibodies used for immunohistochemical analysis with their respective dilutions

3.10 Measure of spontaneous activity in novel environment

The spatial exploration task was carried out as reported previously (Caston et al., 1998) with some modifications. Adult male mice aged P60 were used and experiments were carried out only during the mornings. 24 hours before the start of the experiment, the mice were

habituated to the experimental room and 50cm (length) x50cm (width) x38cm (height) exploration chamber for 30 and 3 minutes each. Each mouse was habituated to the chamber 3 times to reduce stress induced activation. After each session, the box was wiped down with water and 70% ethanol to remove any olfactory cues from the previous animal. On the subsequent day, the experimental mice were habituated to the room for 30 minutes after which they were allowed to explore the chamber for 10 minutes each. Analysis was then carried out to determine the distance travelled and level of relative activity between the control and experimental groups. Video analysis was carried out using Ethowatcher.

3.11 Behavioural analysis

Food deprivation was carried out on male mice aged P60 to P65, all of whom on average weigh between 28-30g at the start. Animals in the experimental group were subjected to 48 hours of food deprivation with a one hour eating period provide at the 24 hour mark. Deprived animals used for analysis all had a 9-10% drop in their respective body weights.

3.12 Image acquisition

All images in this report were captured using either the 10x, 20x or 63x oil objective on the Zeiss LSM-710 Confocal Microscope System (Axio Imager Z2). Images obtained were in an 8-bit format and imaging parameters such as laser power, digital gain and offset were kept unchanged for each experiment. Processing was carried out using ImageJ (Abràmoff et al., 2004) and Fiji (Schindelin et al., 2012).

3.13 Image analysis and quantification

3.13.1 Cell count

Cell counting for food deprivation analysis and *c-Fos* was carried out in the same manner. Each image was opened on ImageJ (Abràmoff et al., 2004) and the multi-channel stacks of 10µm thickness, in steps of 1µm, were compressed to form composite images with calbindin as a marker for Purkinje cells in the food deprivation analysis. The elliptical drawing tool was selected and calbindin positive neurons were manually drawn out in each region of interest and added to the manager after which the results were generated.

3.13.2 Intensity measurement

In order to measure the intensity of *Mef2c* or *cFos* in Purkinje cells, 20x z-stack images of either 10µm or 20µm were used. Images were acquired in steps of 1µm and were compressed before analysis. The threshold for each of these channels was then independently determined as mentioned previously (Harrington et al., 2016) but with slight alterations. The average pixel intensity of at least five non-overlapping regions was first calculated and the standard deviation generated was added to this value. Using the subtract function in ImageJ, background was eliminated from this image. Similar to the method used for cell counting, the elliptical tool was selected and each individual Purkinje cell was drawn out and the intensity was determined for the respective channels. These intensities were then further normalized to account for the disparity in antibody staining from one experiment to the next. Average intensity values were obtained for both the control and experimental Purkinje cells from at least five images taken from 2-3 different slides. The number obtained from dividing average intensity of experimental group versus the control group was used to normalize all the images.

3.13.3 GAD67/vGluT2 puncta quantification

To carry out puncta analysis, 63x oil z-stack images of 10 μ m thickness, in steps of 0.5, were obtained. ImageJ was used to convert it into a maximum intensity stack after which thresholding was carried out as mentioned above to obtain the final images. The GFP channel was retained green while both GAD67 and vGluT2 were converted to red. Using the RG2B plugin on ImageJ, which supports only dual-channel co-localization, yellow puncta was identified within manually drawn region of interest either covering the full area of the soma or the dendrites. Analyze Particle property on Image J was then applied to determine the exact number of puncta in the specified region in both GAD67 and vGluT2 individually and % of synaptic density was determined by dividing number of puncta by area and multiplying it by 100. Normalization was carried out in the same way mentioned above but with average number of particles in control and experimental groups instead of pixel intensity. All further statistical tests were carried out using GraphPad Prism.

3.13.4 Dendrite tracing and analysis

For P14 as well as P21 morphological analysis, 20x stack images of only GFP in 50 μ m and 80 μ m (steps of 1) thickness were obtained respectively. Following the aforementioned method of thresholding, each dendrite of the Purkinje cell tree was traced out manually using the Simple Neurite Tracer (Longair et al., 2011) plugin on Fiji. 3D-Sholl analysis using concentric circles in steps of 10 μ m was conducted using the plugin on ImageJ to obtain data for maximum intersections, radii of maximum intersections and intersection number vs distance from soma, all analyzed on GraphPad.

Figure 3-14: Process of dendritic tracing and analysis. (A) 20x stack image of GFP channel acquired on the confocal. (B) A fully traced neuron using the simple neurite tracer on Fiji. (C) Traces are filled using the Fill Out function to get the final image as shown.

For P14 as well as P21 morphological analysis, 20x stack images of only GFP in 50 μ m and 80 μ m (steps of 1) thickness were obtained respectively. Following the aforementioned method of thresholding, each dendrite of the Purkinje cell tree was traced out manually using the Simple Neurite Tracer (Longair et al., 2011) plugin on Fiji. 3D-Sholl analysis using concentric circles in steps of 10 μ m was conducted using the plugin on ImageJ to obtain data for maximum intersections, radii of maximum intersections and intersection number vs distance from soma, all analyzed on GraphPad.

3.13.5 Spine analysis

A 63x oil immersion was used to take 20 μ m stack images of only the GFP channel. Maximum intensity projections of this stack, taken in steps of 0.5, were generated and threshold values were

calculated as mentioned previously. Images were then converted to greyscale and spines were manually counted from at least 20 independent tertiary/ terminal dendrites using the Cell Counter function on ImageJ. Total number of spines was determined in 10 μ m stretches of dendrite per neuron and density was calculated as number of spines divided by 10. GraphPad was employed for subsequent t-test analysis.

3.14 Statistical analysis

All data obtained was analyzed using GraphPad Prism. Pattern of data distribution was first identified for each data set and outliers were calculated using quartile calculations. Unpaired t-tests were used for all the experiments. Significance values is as follows: * $p < 0.05$, ** $p < 0.01$ or 0.001 or 0.0001, as mentioned in respective figure legends.

4. RESULTS

4.1 Relative levels of *Mef2* transcripts in the neuronal subpopulations of the cerebellar cortex

Although studies have made significant progress in identifying and characterizing the neuronal subpopulations of the cerebellar cortex (Marzban et al., 2015), details of genes that are expressed by specific population of neurons are still lacking. An Affymetrix microarray database generated by our collaborators was analyzed to shortlist gene candidates which showed elevated levels of expression in specific neuronal subtypes in the cerebellar cortex (N. Heintz and B. Barres, unpublished). Cre-driven mouse lines using Purkinje cell protein 2 (PCP2), metabotropic glutamate receptor 2 (mGluR2), LY6/PLAUR domain 6 (LYPD6) and neurogenic differentiation 1 (Neurod1) were used to isolate ribosomes from Purkinje, Golgi, stellate, basket and granule cells respectively.

Gene	Purkinje cell (PCP2)	Golgi cell (mGluR2)	Granule cell (Neurod1)	Stellate and basket cells (Lypd 6)
<i>Mef2a</i>	2522	285	2210	1787
<i>Mef2b</i>	2	2	3	2
<i>Mef2c</i>	5911	32	774	613
<i>Mef2d</i>	371	347	1096	413

Table 4-1: Microarray results showing the relative expression levels of the four *Mef2* genes in each of the neuronal subtypes of the adult cerebellar cortex.

Using results from this microarray, we set out to determine the distribution of all *Mef2* transcription factors in the neuronal subtypes of the cerebellar cortex. Even though the

distribution of *Mef2* transcripts has been previously characterized in the CNS, the study did not determine the specific layers and neuronal subpopulations that express each of these genes in the cerebellum (Lyons et al., 1995). Therefore, results from this microarray was informative in addressing both the distribution of *Mef2* transcripts in specific regions and neuronal subtypes of the cerebellar cortex as well as their relative expression levels.

According to the microarray results shown in **Table 4-1**, *Mef2a*, *Mef2c* and *Mef2d* are differentially expressed in the neurons of the cerebellar cortex unlike *Mef2b* which was not detectable in all neuronal subtypes. Both *Mef2a* and *Mef2d*, which have been previously reported to work as heterodimers are enriched in all four neuronal subtypes, with *Mef2a* showing a higher level of expression than *Mef2d* (**Table 4-1**) (Zhao et al., 1999). On the other hand, *Mef2c* transcripts were found in almost negligible amounts in the Golgi cells, moderate levels in the granule, stellate and basket cells and robust expression in Purkinje cells. Apart from the roles of *Mef2a* and *Mef2d* in the survival of cerebellar granule neurons (Shalizi and Bonni, 2005), very little is known about the function of *Mef2* in the cerebellar cortex. Furthermore, the function of *Mef2c* has not yet been examined in the cerebellum. Therefore, data from this microarray would be a useful in verifying further characterization of unique genes such as the *Mef2* transcription factors and help us in better understanding their functions in the cerebellum.

4.2 Analysis of the RNA expression profile of *Mef2*

In order to assess the validity of the microarray, specific probes for each of the *Mef2* genes were designed and used to perform *in situ* hybridization analysis. In all three mice analyzed, expression of *Mef2a* and *Mef2d* RNA was found in the ML, PL and IGL of the cerebellar cortex, consistent with the results from the microarray suggesting their presence in Golgi, granule, stellate-basket cells and Purkinje cells (**Figure 4-2B, F**). *Mef2b* expression, also

consistent with the microarray data, showed no expression in any of the layers of the cerebellar cortex (**Figure 4-2, C**). However, analysis of the RNA expression of *Mef2c* revealed a high level in the PL, but no expression was detected in the IGL or the ML as suggested otherwise in the microarray (**Figure 4-2, D**). Analysis of *Mef2c* expression in the human and mouse brain also indicates that expression of *Mef2c* was higher in the PL with almost no expression in the IGL and ML (Leifer et al., 1994, Lyons et al., 1995). Together, we show that unlike *Mef2a* or *Mef2d*, *Mef2c* RNA is exclusively expressed in the PL.

Figure 4-2: *Mef2a*, *Mef2c* and *Mef2d* are differentially expressed in the three layers of the adult cerebellar cortex. (A) Schematic diagram showing the sagittal section of the cerebellum. Red box indicates the region of cerebellar lobule VIII from which images were obtained. (B) *Mef2a* RNA was detected in the ML, PL and IGL of the cerebellar cortex. (C) No expression of *Mef2b* RNA was detected in all three layers. (D) Model of the cerebellar cortex showing the ML, PL and IGL. (E) *Mef2c* transcripts were found exclusively in the PL; no expression was detected in the ML or the IGL. (F) Expression of *Mef2d* was detected in the ML, PL and IGL layers. For all figures, n=3 mice. Scale bar= 20 μ m. Abbreviations: ML= molecular layer. PL/Purkinje cell= Purkinje cell layer, IGL =Internal granule layer.

4.3 Characterization of the expression of *Mef2c* protein in the cerebellar cortex

4.3.1 *Mef2c* is expressed exclusively by the Purkinje cells of the cerebellum

Both the microarray data as well as the *in situ* hybridization results revealed that *Mef2c* expression is restricted to the Purkinje layer of the cerebellar cortex, strongly suggesting the presence of *Mef2c* in Purkinje cells. To confirm this, co-localization analysis was carried out with known molecular markers of cerebellar neuronal subtypes. Double immunostaining with *Mef2c* and Calbindin, a well-established marker for Purkinje cells in the cerebellar cortex, confirmed that *Mef2c* was indeed present in this subset of cerebellar neurons (**Figure 4-3-1, A-D**); (Vecellio et al., 2000). Although *Mef2c* RNA was undetectable in the ML as well as the IGL, it was necessary to verify that *Mef2c* protein expression also displayed similar distribution patterns. When *Mef2c* was co-stained with Parvalbumin, a marker for Purkinje, stellate and basket cells, immunopositive nuclei were only detected in the PVA positive cells in the PL and absent in the neurons in the ML (**Figure 4-3-1, E-H**); (Vecellio et al., 2000). Not only did this observation show that *Mef2c* is not expressed in stellate and basket cells, but it also further validated the presence of *Mef2c* in the Purkinje cells. Similar staining assays with mGluR2 and NeuN which respectively mark Golgi (**Figure 4-3-1, I-L**) and granule cells (**Figure 4-3-1, M-P**); (Neki et al., 1996, Weyer and Schilling, 2003), showed no overlap with *Mef2c*, validating its absence in these neurons. Taken together, we determined *Mef2c* expression is restricted to Purkinje cells in the cerebellar cortex and identified a novel molecular marker for this cerebellar neuronal subtype.

Figure 4-3-1: *Mef2c* protein expression is limited to Purkinje cells in the adult cerebellar cortex. (A-D) Schematic diagram represents the layers of the cerebellar cortex. Objects highlighted in green in each panel represents the neuronal subpopulation that is marked in the green panel along the same row. (B-D) Co-localization of *Mef2c* with calbindin, a marker for Purkinje cells in the cerebellum, confirms the expression of *Mef2c* in Purkinje cells. (F-H) Parvalbumin, a marker for stellate and basket cells in the ML and Purkinje cells in the PL shows *Mef2c* co-localization only within the Purkinje cells and not within the neurons in the ML. (J-L) mGluR2, a marker for Golgi cells in the IGL, does not show any overlap with *Mef2c* indicating that Golgi cells do not express *Mef2c*. (N-P) No co-localization was detected when *Mef2c* is merged with NeuN, a marker for granule cells and a small population of stellate and basket cells in the ML. All images were taken from cerebellar lobule VIII, n=3 mice, all aged between P60-

P63. Scale bar =10µm. Abbreviations: ML= molecular layer. PL= Purkinje cell layer, IGL =Internal granule layer

4.3.2 Mef2c expression in Purkinje cells does not follow Zebrin-defined patterns

The cerebellum is known to have functionally exclusive regions defined by the presence or absence of Zebrin in the Purkinje cells of a particular microzone (Hawkes and Herrup, 1995). Since we have provided evidence that Mef2c is expressed by Purkinje cells, we next set out to determine if Mef2c expression in Purkinje neurons follows Zebrin-defined patterns. Using double immunohistochemistry, we carried out a co-localization analysis of Mef2c with Zebrin. A small subset of Purkinje cells was found to co-localize with Zebrin in both the apical as well as the basal region suggesting that *Mef2c* was expressed by all Purkinje cells in this region regardless of the status of Zebrin expression (**Figure 4-3-2, C-H**). Analysis of the colocalization of Mef2c with Calbindin, which marks Purkinje cells in the cerebellum, similarly showed complete co-localization (**Figure 4-3-2, I-K**). Hence, our results indicate that Mef2c gene is a general Purkinje cell-specific marker that does not delineate cerebellar microzones.

Figure 4-3-2: Mef2c is expressed by all Purkinje cells in the mature cerebellum. (A) Coronal section of the cerebellum stained with Mef2c (red). White boxes indicate apical and basal regions featured in panel C and D. (B) Schematic diagram showing the coronal section of the cerebellum. (C-H) Co-expression of Mef2c and Zebrin show that Mef2c is present all Purkinje cells, regardless of their Zebrin profile. (I-K) Double immunolabelling with calbindin, taken from the apical region further confirms the presence of Mef2c in all Purkinje cells. For all figures, n=3 mice. Scale bar = 10µm. Abbreviations: AP= Apical, BA= Basal.

4.3.3 Onset of Mef2c expression occurs in the early postnatal stages of Purkinje cell development

Several genes that play important regulatory roles in cerebellar development have been reported to have transient expression patterns (Wang and Zoghbi, 2001). We have provided evidence that Mef2c is expressed exclusively by the Purkinje cells in the adult brain but its developmental expression pattern has not been determined. Mef2c was absent at both E15.5 and E18.5 in the embryonic cerebellum (**Figures 4-3-3, A-F**). However, Mef2c expression is detected at P0, suggesting that the onset of Mef2c occurs somewhere in the time frame between E18.5 and P0 (**Figures 4-3-3, G-I**). Once detected at P0, Mef2c remains expressed at P7, P14 and P21 (**Figures 4-3-3, J-L, M-O, P-R**). With the results of this analysis, we conclude that Mef2c might have important roles to play during specific developmental milestones of Purkinje cell development.

Mef2c/Calbindin

Figure 4-3-3: Mef2c expression comes on during early postnatal stages and is maintained in adulthood. (A, D, G, J, M, P) Schematic diagram A and D represent the structure of the embryonic cerebellum at E15.5 and E18.5 respectively. The models in G, J, M and Q show the cerebellar cortex with its layers. Blue cells in all the models represent calbindin while the red cells show Mef2c. (A-C) At E15.5 expression of calbindin (blue) is detected in the VZ and WM. Mef2c (red) is absent. (D-F) At E18.5 calbindin positive cells persist and move towards the EGL. Mef2c expression is still undetected. (G-I) P0 shows the onset of Mef2c expression in the calbindin positive Purkinje cells in the immature PL. (J-R) Mef2c expression persists in 7-day old Purkinje cells (J-L) which then continues to P14 (M-O) and P21 (P-R). All images were taken from cerebellar lobule VIII, n=3 mice for each age group. Scale bar =10µm. Abbreviations: EGL= external granule layer, WM= white matter, VZ= ventricular zone, ML= molecular layer, PL= Purkinje cell layer, IGL =Internal granule layer.

4.4 Determining the efficiency of L7 driven lentivirus in knocking down *Mef2c* expression

Purkinje cells undergo dynamic morphological changes during early postnatal stages which coincide with the onset of *Mef2c* expression (Sotelo and Dusart, 2009). This suggests that *Mef2c* may contribute to aspects of the development and maturation of Purkinje cells. In order to explore this possibility, we first developed a lentiviral tool for the specific and efficient knockdown of *Mef2c* selectively in the Purkinje cells. A previous study successfully employed the use of a minimal region of the L7 gene as a Purkinje cell specific promoter (Sawada et al., 2010). Short-hairpin RNA (shRNA) sequences were designed and generated for control (scrambled) as well as *Mef2c* knockdown, and placed downstream of the truncated L7-CMV promoter as shown in **Figure 4-6, A**. Lentiviral particles plasmid expressing a green fluorescent protein (GFP) reporter were then generated and transduction of Purkinje cells was carried out at different stages during Purkinje cell development (**Figure 4-6, B**). Viral infection carried out on P1 pups sacrificed at P21 results in specific and strong expression of GFP in Purkinje cells

(Figure 4-6, C-D). In order to determine the efficiency of transduction, tissues from infected pups were subjected to free floating IHC with *Mef2c* (red) and GFP (green) and images acquired were used to calculate intensity differences between the scrambled and knockdown groups (Figure 4-4, E-J). The level of *Mef2c* expression in the knockdown group was ~3 fold lower than the control (Figure 4-4, K). With this result, we show that the virus carrying shRNA plasmids with the L7 minimal promoter permits both specific and efficient knockdown of *Mef2c* and is a potent tool to analyze the function of *Mef2c*.

Figure 4-4: Specific and efficient lentiviral knockdown of *Mef2c* in Purkinje cells using a minimal L7 promoter. (A) Schematic diagram showing the constructs used to generate lentiviral particles. L7-CMV (grey) is the promoter driving expression of control and *Mef2c* knockdown shRNAs (red) and GFP (green). An IRES site (black) and miRNA cassette (blue) was also juxtaposed in between. (B) Timeline of Purkinje cell development showing the contrasting morphologies of Purkinje cells at specific time points. (C-D) Tile scans of the sagittal

sections showing the infectivity of the control (shScrambled) as well as the knockdown (sh*Mef2c*) viruses in lobule X of the cerebellum. (E-J) IHC of Purkinje cells infected at P1 and scarified at P21 with control and knockdown viruses expressing GFP (green) show a notable reduction in *Mef2c* (red) levels in the latter group. Free-floating IHC, section thickness 100µm. (K) Graph showing ~3 fold reduction in the levels of *Mef2c* in the knockdown group, relative to control. ** $p > 0.001$. n= 20 neurons from 4 brains for scrambled, n=17 neurons from 6 brains for *Mef2c* knockdown. Purkinje cells analyzed were taken from lobules III to VIII. Scale bar= 500µm for tile scans, 20µm for panel D. Abbreviations: CMV= cytomegalovirus, IR= IRES, GFP =green florescence protein, sh= short-hairpin, Scr = Scrambled.

4.5 Consequences of viral transduction on viability of Purkinje cells

Genetic manipulation of neurons sometimes results in cell death (Thomas et al., 2003). Therefore, it is essential to determine the viability of infected cells before carrying out further analysis. Previous reported have shown that Purkinje cells are particularly vulnerable at P3 which is therefore an ideal stage to determine neuronal viability upon transduction (Wassef et al., 1987). Scrambled and *Mef2c* knockdown viruses were introduced into the cerebellum of P3 pups, and harvested at P14 (**Figure 4-5, A**). The levels of GFAP, a marker for Bergmann glia in the cerebellum, surrounding Purkinje cell soma is elevated upon inflammation, injury and cell death (Marín-Teva et al., 2004). Co-staining of GFP with GFAP did not shown any increase in GFAP expression in the soma of Purkinje cells in the control and knockdown groups (**Figure 4-5, B-M**). Another characteristic of Purkinje cell death is the shrinking of their soma (Valkova et al., 2017). The size of control and knockdown Purkinje cells were measured manually and values were compared using t-test on GraphPad prism. The size of Purkinje cell somata in both control and knockdown groups was comparable suggesting that loss of *Mef2c* does not result in Purkinje cell death (**Figure 4-5, N**) (Valkova et al., 2017).

Figure 4-5: L7-driven lentiviral knockdown of *Mef2c* does not affect the viability of Purkinje cells. (A) Schematic diagram showing the Purkinje cell developmental timeline. Red boxes at P3 and P14 indicate stages of viral transduction and brain harvest respectively. (B-M) Panels showing co-localization of GFP (green) and GFAP (red) in P14 Purkinje cells. (N) Graph showing soma size in both control and knockdown groups. $p=0.8896$. $n= 14$ neurons from 4 brains for scrambled, $n=18$ neurons from 5 brains for *Mef2c* knockdown. Purkinje cells analyzed were taken from lobules III to VIII. Scale bar = $5\mu\text{m}$. Abbreviations: GFAP= Glial fibrillary acidic protein, GFP =green florescence protein, sh= short-hairpin.

4.6 Consequences of *Mef2c* knockdown on Purkinje cell migration patterns

Purkinje cells are generated from E10.5 to E12.5 after which they continue migrating until P15-P18 (Altman and Bayer, 1978). Purkinje cells of P0- P1 mice are found in multi-layers within the PL-IGL boundary which continue migrating until they form a monolayer in the Purkinje cell layer between P0 to P4 (**Figure 4-6, A**). Analysis of Purkinje cells with the loss of *RORα* has shown the presence of some Purkinje cells in the IGL, indicating a problem with the migration process (Takeo et al, 2015). To determine the localization pattern of Purkinje cells after *Mef2c* knockdown, we performed double labelling of calbindin with GFP. We observe that knockdown of *Mef2c* in Purkinje cells does not alter the formation of a monolayer alignment of Purkinje cells in the Purkinje layer from P0 to P4 (**Figure 4-6, Bi-Bvi**).

Figure 4-6: Loss of *Mef2c* in developing Purkinje cells does not affect migration. (A)

Schematic diagram showing multi-layers Purkinje cells at P0 which, upon completing migration at P21, form a monolayer in the Purkinje cell. (B) Panels showing co-localization of GFP (green) and GFAP (red) in P14 Purkinje cells. Purkinje cells analyzed were taken from lobules III to VIII, n= 10 neuron from 2 brains for each group. Scale bar = 5 μ m. Abbreviations: Purkinje cell= Purkinje cell layer, GFP =green florescence protein, sh= short-hairpin.

4.7 Determining the possible roles of *Mef2c* in the elimination of somatic dendrites on Purkinje cells

When Purkinje cells transition from stellate-shaped cells at P4 to an immature Purkinje cell at P8, they undergo pruning of processes on the soma (Sotelo and Dusart, 2009). The loss of some transcription factors such as *ROR α* in developing Purkinje cells has been shown to impact the elimination somatic processes during the pruning phase from P3 to P7 (Takeo et al., 2015). In order to determine if *Mef2c* might contribute to this process of elimination, we transduced P3 Purkinje cells, harvested them at P14 (**Figure 4-7, A**). Soma of GFP positive neurons were analyzed for short dendritic protrusions that have not been pruned. Purkinje cells in both shScrambled and sh*Mef2c* did not show any remnant processes on their soma indicating that *Mef2c* does not appear to control the process of dendritic elimination in developing Purkinje cells (**Figure 4-7, B**).

Figure 4-7: Loss of *Mef2c* at P3 does not disrupt the elimination of somatic dendrites. (A) Schematic diagram showing the Purkinje cell developmental time. Virus was introduced into P3 cerebelli and brains were harvested at P14. (B-C) Soma of transduced Purkinje cells from control as well as knockdown conditions. Purkinje cells analyzed were taken from lobules III to VIII, n=13 from 4 brains for shScrambled and n= 19 from 5 brains for sh*Mef2c*. Scale bar = 10µm.

4.8 Investigating the possible roles of *Mef2c* in Purkinje cell spinogenesis

Early postnatal spinogenesis is one of the processes in Purkinje cell development that is controlled primarily by intrinsic factors (Bravin et al., 1999, Sotelo and Dusart, 2009). In order to determine if the loss of *Mef2c* might affect spine formation on Purkinje cells, P3 mice were transduced with virus and the spines of GFP positive neuron at P14 were analyzed (**Figure 4-8, A**). Purkinje cells are known to possess two types of spines, namely thorns and spiny branchlets (Sotelo, 1975). In this analysis we only study changes in number of spiny branchlets present in the distal parts of the Purkinje cells **Figure 4-8, B**. Loss of *Mef2c* in developing Purkinje cells showed an ~12 % increase in spine numbers compared to control (**Figure 4-8, C-E**). Our results indicate that *Mef2c* could be one contributor to the process of spinogenesis during Purkinje cell development.

Figure 4-8: *Mef2c* negatively regulate spinogenesis in developing Purkinje cells. (A) Schematic diagram showing the Purkinje cell developmental time. Virus was introduced into P3 cerebelli and brains were harvested at P14. (B) Schematic diagram showing the sampling regions (red) for each Purkinje cell analyzed. (C-D) Binary images of GFP infected Purkinje cells from control as well as knockdown groups. (E) Graph showing the relative increase in spine densities of Purkinje cells in the knockdown group. Purkinje cells analyzed were taken from lobules III to VIII, spine counts were carried out across 20 segments, each of 10μm length, taken from 1 neuron, n=3 from 3 brains each for shScrambled and sh*Mef2c*. Scale bar = 5μm.

4.9 Analyzing the effects of P1 *Mef2c* knockdown on morphology of P14 Purkinje cells

4.9.1 Knockdown of *Mef2c* results in the increase of dendritic intersections of 14-day-old

Purkinje cells

Several mouse models for autism, ataxia and other neurodevelopmental disorders exhibit a loss in number or severe morphological alterations of Purkinje cells (Valkova et al., 2017). Morphological properties reveal important aspects of Purkinje cells as excitatory and inhibitory inputs synapse on the soma and dendrites, assisting the normal functioning of these neurons (McKay and Turner, 2005). P1 Purkinje cells were transduced with either control or knockdown viruses and brains were harvested at P14 (**Figure 4-9-1, A**). The dendrites of GFP positive neurons were traced as described in methods and Sholl analysis was performed on the tracings. Representative images of reconstructed dendrites of Purkinje cells for each treatment group are shown in **Figure 4-9-1, B**. Loss of *Mef2c* results in a 65% increase in the total number of intersections made by Purkinje cell dendrites implying that *Mef2c* regulates the growth of dendrites (**Figure 4-9-1, C**). Additionally, the radius at which maximum intersections were observed was 56% more distal to the soma for sh*Mef2c* than shScrambled (**Figure 4-9-1, D**). In spite of a sharp increase in dendritic number in the knockdown group, the height of Purkinje cells, analyzed by measuring the maximum length of the secondary dendrites, showed no significant difference from that in the control (**Figure 4-9-1, E**). Also, a comparison of the distribution of dendritic intersections revealed that Purkinje cells from both groups show increased dendritic growth beyond the 60 μ m radius with sh*Mef2c* neurons showing a sharper increase at the outer boundary of the Purkinje cells (**Figure 4-9-1, F**). Cumulatively, these set of results show that *Mef2c* play specific roles in shaping the dendritic morphology of Purkinje cells from P1 to P14.

Figure 4-9-1: Loss of *Mef2c* increases dendritic growth of Purkinje cells from P1 to P14. (A) Schematic diagram showing the Purkinje cell developmental time. Virus was introduced into P1 cerebelli and brains were harvested at P14. (B) Figure panel showing reconstructed neurons from scrambled and knockdown groups. (C) Graph showing the comparison of the total number of intersections between the shScrambled and sh*Mef2c* treatments. There was a significant increase in the number of intersections in Purkinje cells in the knockdown group versus in the control groups, ** $p=0.0063$. (D) Radius of maximum intersection graph showing that Purkinje cells with the loss of *Mef2c* show a longer radius when compared to the Purkinje cells of the control

group, $**p = 0.0022$. (E) Height of Purkinje cells in both the control and knockdown groups remain unchanged, $p = \text{ns}$. (F) A plot of intersections relative to the distance from the soma showing larger number of intersection in sh*Mef2c* vs shScrambled beyond the 60 μm radius. Purkinje cells analyzed were taken from lobules III to VIII, $n=11$ from 3 brains for shScrambled and $n= 18$ from 5 brains for sh*Mef2c*. Scale bar = 20 μm .

4.9.2 Analyzing the effects of P1 Mef2c knockdown on morphology of mature Purkinje cells

Between P14 and P21, Purkinje cells undergo a sharp increase in the dendritic growth that corresponds to other changes such as completion of synapse elimination and formation of functional GABAergic synapses by stellate and basket cells (Sotelo and Dusart, 2009). In order to better understand possible contributions of *Mef2c* in Purkinje cell development beyond P14, we transduced P1 pups and analyzed the morphology of P21 transfected neurons (**Figure 4-9-2, A, B**). Similar to the observations at P14, we observed a 1.7 fold increase of dendrites in P21 Purkinje cells infected with sh*Mef2c* compared to control (**Figure 4-9-2, C**). Even at P21, the relative height of Purkinje cells from both groups remains comparable (**Figure 4-9-2, D**).

However, as shown in **Figures 4-9-2, D, F**, the difference in radius of maximum intersection and overall distribution of intersections relative to the soma show no significant differences, unlike what was observed at P14 (**Figure 4-9-2, C-F**). Taken together, these results indicate that while *Mef2c* seems to increase dendritic intersections at both P14 and P21, there seems to be a higher number of dendrites in the proximal region at P21 than at P14. Therefore, it appears that *Mef2c* controls morphological properties of dendrites in an age-dependent manner.

Figure 4-9-2: Loss of *Mef2c* results in additional increase in dendritic growth of Purkinje cells from P1 to P21. (A) Schematic diagram showing the Purkinje cell developmental time. Virus was introduced into P1 cerebelli and brains were harvested at P21. (B) Figure panel showing reconstructed neurons from scrambled and knockdown groups. (C) Graph showing the comparison of the total number of intersections between the shScrambled and sh*Mef2c* treatments. There was a significant increase in the number of intersections in Purkinje cells in the knockdown group versus in the control groups, ** $p = 0.005$. (D) Radius of maximum intersection

graph showing no significant difference in radii of maximum intersections. (E) Height of Purkinje cells in both the control and knockdown groups remain unchanged. (F) A plot of intersections relative to the distance from the soma showing no significant differences in distribution of intersections on P21 Purkinje cells. Purkinje cells analyzed were taken from lobules III to VIII, n=11 from 3 brains for shScrambled and n= 18 from 5 brains for sh*Mef2c*. Scale bar = 20µm.

4.9.3 Analyzing the effects of P7 Mef2c knockdown on morphology of mature Purkinje cells

Purkinje cell development is controlled by both intrinsic as well as extrinsic factors (Sotelo and Dusart, 2009). The neurodevelopmental processes that occur from P0 to P7 are primarily controlled by intrinsic factors (Sotelo and Dusart, 2009) beyond which it is a combination of intrinsic and extrinsic factors that modulate Purkinje cell development (Tavosanis, 2012). To gain more insight into on whether intrinsic factors alone can alter Purkinje cell morphogenesis beyond P7, we knockdown *Mef2c* from mice at the end of the first postnatal week and analyze them at P21 (**Figure 4-9-3, A-B**). Interestingly, we observed that loss of *Mef2c* in Purkinje cells from P7 to P21 showed 33% increase in number of intersections but other variables such as height of Purkinje cells or radius and distributions of dendritic intersections remain unchanged (**Figure 4-9-3, D-G**). Our results therefore suggest that even in the presence of extrinsic factors, the loss *Mef2c* altered dendritogenesis indicating that it carries out essential roles in the development and maturation of Purkinje cells in the first three postnatal weeks.

Figure 4-9-3: P7 knockdown of *Mef2c* showed an increase in dendritic intersections in mature Purkinje cells. (A) Schematic diagram showing the Purkinje cell developmental time. Virus was introduced into P7 cerebelli and brains were harvested at P21. (B-C) Figure panel showing reconstructed neurons from scrambled and knockdown groups. (D) Graph showing the comparison of the total number of intersections between the shScrambled and sh*Mef2c* treatments. There was a significant increase in the number of intersection between the groups. (E) Graph depicting the relative radii of maximum intersection of shScrambled and sh*Mef2c* show that points of maximum intersections were not found in significant distance of each other. (F) Height of Purkinje cells in both the control and knockdown groups remain unchanged. (F) A plot of intersections relative to the distance from the soma showing similar trends of intersection distribution in control as well as knockdown groups. Purkinje cells analyzed were taken from

lobules III to VIII, n= 8 from 3 brains for shScrambled and n= 6 from 3 brains for sh*Mef2c*.
Scale bar = 20µm

4. 10 Examining the effects of P1 *Mef2c* knockdown on the GAD67-vGluT2 profile in developing Purkinje cells

4.10.1 Purkinje cell-specific loss of Mef2c from P1 to P14 results in an increase in GAD67 synapses

Loss of *Mef2c* in neurons of the somatosensory cortex results in an imbalance of excitatory and inhibitory synapse densities during development (Harrington et al., 2016). In order to determine whether *Mef2c* plays a similar role in the cerebellum, Purkinje cells were transduced at P1 and the brains were harvested at P14. GAD67, a marker for GABAergic synaptic puncta and vGluT2, a marker for glutamatergic climbing fiber synaptic inputs were co stained with GFP from the virus and analyzed as discussed in the methods section (Martin and Barke, 1998, Miyazaki et al., 2003). Knockdown of *Mef2c* results in an increase the number of GAD67 synapses in the somatic and dendritic compartments of Purkinje cells at P14 (**Figure 4-10-1, B, D, F, G**). Unlike GAD67, vGluT2 puncta showed no change in the somatic compartment (**Figure 4-10-1, C, E, H**).

Figure 4-10-1: Knockdown of *Mef2c* results in an increase in the number of GAD67 puncta without affecting vGluT2 in 14 day old Purkinje cells.

(A) Schematic diagram showing the Purkinje cell developmental time. Virus was introduced into P1 cerebelli and brains were harvested at P14. (B) Cropped images showing the distribution of GAD67 puncta (red) in the Purkinje cell dendrites (Bi, iii, v) and soma (Bii, iv, vi) transfected with shScrambled (GFP). (C) Cropped images showing the distribution of vGluT2 puncta (red) in the Purkinje cell dendrites (Ci, iii, v) and soma (Cii, iv, vi) transfected with shScrambled (GFP). (D) Cropped images showing the distribution of GAD67 puncta (red) in the Purkinje cell dendrites (Di, iii, v) and soma (Dii, iv, vi) transfected with sh*Mef2c* (GFP). (E) Cropped images showing the distribution of vGluT2 puncta (red) in the Purkinje cell dendrites (Ei, iii, v) and soma (Eii, iv, vi) transfected with shScrambled (GFP). (F-G) Graphs showing a significant reduction of GAD67 density in Purkinje cell soma. * $p=0.0390$ and dendrites. * $p=0.0453$. (H-I) Graphs showing no significant changes in vGluT2 puncta densities in both Purkinje cell soma and dendrites. $p=ns$. Purkinje cells analyzed were taken from lobules III to VIII, $n=14$ neuron from 4 brains for shScrambled and $n=16$ from 7 brains for sh*Mef2c*. Scale bar = $5\mu m$. Abbreviations: GFP =green florescence protein, sh= short-hairpin.

4.10.2 Mef2c knockdown on the GAD67-vGluT2 profile in P21 Purkinje cells

P14 to P21 is a critical window of Purkinje cell development during which there is a sharp rise in both excitatory and inhibitory inputs onto cerebellar Purkinje cells (Hockberger et al., 1989). Analysis of the expression of GAD67 and vGluT2 on P21 Purkinje cells lost *Mef2c* expression at P1, revealed slightly different trends when compared to the analysis at P14. Although the synaptic density of GAD67 on the soma is reduced at P21, there was no significant reduction of GAD67 on the dendrites (**Figure 4-10-2, B, D, F, G**). Interestingly, the number of vGluT2 synaptic puncta that were unchanged at P14 in the dendrites showed a 58% decrease at P21. (**Figure 4-10-2, C, E, I**). No changes vGluT2 was observed in the somatic compartment (**Figure 4-10-2, C, E, H**).

Figure 4-10-2: Knockdown of *Mef2c* shows a reduction in the number of GAD67 and vGluT2 puncta in the soma and dendrites in P21 Purkinje cells respectively

(A) Schematic diagram showing the Purkinje cell developmental time. Virus was introduced into P1 cerebelli and brains were harvested at P21. (B) Cropped images showing the distribution of GAD67 puncta (red) in the Purkinje cell dendrites (Bi, iii, v) and soma (Bii, iv, vi) transfected with shScrambled (GFP). (C) Cropped images showing the distribution of vGluT2 puncta (red) in the Purkinje cell dendrites (Ci, iii, v) and soma (Cii, iv, vi) transfected with shScrambled (GFP). (D) Cropped images showing the distribution of GAD67 puncta (red) in the Purkinje cell dendrites (Di, iii, v) and soma (Dii, iv, vi) transfected with sh*Mef2c* (GFP). (E) Cropped images showing the distribution of vGluT2 puncta (red) in the Purkinje cell dendrites (Ei, iii, v) and soma (Eii, iv, vi) transfected with shScrambled (GFP). (F-G) Graphs showing a significant reduction of GAD67 density in Purkinje cell soma, $*p = 0.0430$. GAD67 levels in dendrites also showed a reduction this was not significant, $p = \text{ns}$. (H-I) Graphs showing a significant reduction of vGluT2 puncta density in Purkinje cell dendrites, $*p = 0.0433$. No notable changes were observed in the levels of vGluT2 in the dendrites, $p = \text{ns}$. Purkinje cells analyzed were taken from lobules III to VIII, $n = 14$ neuron from 4 brains for shScrambled and $n = 10$ from 4 brains for sh*Mef2c*. Scale bar = $5\mu\text{m}$. Abbreviations: GFP = green fluorescence protein, sh = short-hairpin.

4.11 Assessing the consequences of behavioural manipulation on *Mef2c* expression

4.11.1 Food deprivation results in the increase of locomotion and neural activity in the hypothalamus

Chronic food deprivation results in an increase in spontaneous locomotor activity in rats (Koubi et al., 1991). AGRP neurons in the hypothalamus exhibit an increase in *cFos* upon food deprivation indicating an increase in neuronal activity (Betley et al., 2013). Mice were food deprived for 48 hours with a 1 hour feeding interval at 24 hours (**Figure 4-11-1, A**). They were then assessed for their spontaneous locomotor activity for a period of 5 minutes in the novel

environment and total distance travelled was measured and plotted as shown in **Figure 4-11-1, B-C**. Double IHC with *cFos* and DAPI showed an increase in the number of Fos positive nuclei in the DMHN of the hypothalamus indicating that an increase in neuronal activity upon food deprivation (**Figure 4-11-1, D-I**). Quantitative analysis of Fos positive nuclei in deprived mice show an significant increase in number of positive nuclei compared to control mice (**Figure 4-11-1, J**).

Figure 4-11-1: Food deprivation results in increase of spontaneous locomotor and neuronal activity. (A) Schematic diagram showing the timeline of food deprivation. Mice were food deprived for 48 hours with a one hour feeding interval at the 24 hours mark. (B) Exploration chamber was used to determine distance travelled and calculate spontaneous activity (N.dep vs Dep) (C) Graph showing a significant increase in total distance travelled in the deprived mice versus the non-deprived mice (D-E) Schematic depicting the nuclei of the hypothalamus with the DMHN in red. (F-I) Measure of *cFos* positive nuclei in DMHN of food deprived and not deprived mice. (J) A plot of the number of *cFos* positive nuclei in the not deprived and deprived groups showing an increase in Fos-positive nuclei upon deprivation. Scale bar for panel D-E= 200μm and F-I= 20μm. Abbreviations: 3V= Third ventricle, DMHN=Dorso-medial hypothalamic nucleus, VMHN=Ventro-medial hypothalamic nucleus, Arc= Arcuate nucleus. NDP= Not deprived, DP= Deprived.

*4.11.2 Behavioral manipulation results in the increase of *Mef2c* expression in the cerebellum*

Level of *Mef2* family of transcription factors in hippocampal neurons increases when mice are subjected to behavioural manipulation such as exploration in a novel environment (Flavell et al., 2008). However, changes in the expression of *Mef2c* in GABAergic neurons after completion of behavioral tasks, has yet to be characterized, particularly in GABAergic neurons. Adult male mice were food deprived for 48 hours with a 1 hour feeding interval at 24 hours (**Figure 4-11-2, A**). Coronal sections of the cerebellum were then stained for *Mef2c* and *cFos* and individual Purkinje cells in CrusI, CrusII and simple lobule of the cerebellum were analyzed (**Figure 4-11-2, B**). In all three regions examined, the intensity of both *Mef2c* and c-Fos are significantly elevated in the deprived group, relative to the fed group as shown in **Figures 4-11-2, E-G** and graphs **G-L**.

Figure 4-11-2: Food deprivation results in an increase in *Mef2c* expression in all regions of the cerebellum. (A) Schematic diagram showing the timeline of food deprivation. Mice were food deprived for 48 hours with a one hour feeding interval at the 24 hours mark. X.

(B) Model of a coronal section of the cerebellum showing the locations of the simple, CrusI and CrusII lobules. (C-D) Tiled images of not deprived and deprived cerebellar coronal sections showing co-localized images of *Mef2c* (red) and *cFos* (blue). (E-G) Cropped images of Purkinje cells from simple, crusI and crusII lobules of the cerebellum. (G-L) Graphs showing the levels of *Mef2c* and *cFos* in the simple, crusI and crusII regions in food deprived and not deprived conditions. Purkinje cells analyzed were taken from simple, crusI and crusII regions,

approximately 70-180 Purkinje cells were counted per region, n=3 each for deprived and not deprived conditions. Scale bars: C-D= 200 μ m, E-G= 20 μ m, **p<0.001 for all graphs.

Abbreviations: SIM= simple lobule, DCN= deep cerebellar nuclei

5. DISCUSSION

Mef2c has been identified as a potential therapeutic target for ASD, schizophrenia and other neurological conditions but very little is known about its distribution and functions in the cerebellum. Analysis of the RNA and protein expression profile of *Mef2* family of transcription factors in the cerebellar cortex revealed that the expression of *Mef2c* is restricted to nuclei of Purkinje cells (**Figures 4-2, 4-3-1, 4-3-2**). The onset of *Mef2c* expression in Purkinje cells between E18.5 and P0 suggests possible involvement in early postnatal Purkinje cell development (**Figure 4-3-3**). Using a Purkinje cell-specific promoter driving the expression of shRNAs against *Mef2c*, we investigated the effects of loss of *Mef2c* at specific time points of Purkinje cell development (**Figure 4-4**). Purkinje cells with reduced *Mef2c* expression did not show cell death, deficits in monolayer alignment or abnormal pruning of somatic dendrites (**Figure 4-5, 4-6, 4-7**).

Knockdown of *Mef2c* at P3, however, showed increased spine density in P14 Purkinje cells (**Figure 4-8**). Additionally, the sustained suppression of *Mef2c* from P1 to the first and second postnatal weeks showed a significant increase in the number of dendritic intersections at both stages but the increase in the number of distal intersections was sharper at P14 than at P21 (**Figure 4-9-1, 4-9-2**). Interestingly, we also observed that silencing the expression of *Mef2c* at a later stage of P7 did not significantly alter the morphology of mature Purkinje cells at P21 indicating that there is a critical period for the influence of *Mef2c* on Purkinje cell morphogenesis (**Figure 4-9-3**).

Analysis of the inhibitory and excitatory synaptic profile as a consequence of the continuous silencing of *Mef2c* from P1 to P14 showed a significant increase in the number of GAD67 puncta in the soma and dendrites (**Figure 4-10-1**). However, by P21, a reduction in

somatic GAD67 and dendritic vGluT2 was observed in Purkinje cells from the knockdown group, implying age-dependent roles for *Mef2c* in reorganizing synapses on Purkinje cells (**Figure 4-10-2**). Apart from this, we also analyzed the expression patterns of *Mef2c* in relatively active versus inactive mice in an effort to better understand the activity-dependent expression of *Mef2c* in specific cerebellar regions. Results revealed that the expression of *Mef2c*, in response to Purkinje cell activation, increases and occurs uniformly across the cerebellum (**Figure 4-11-2**). Taken together, our findings suggest that *Mef2c* regulates important aspects of morphological and synaptic organization during the early stages of Purkinje cell development.

5.1 Distinct roles of each *Mef2* gene homologue in the neurons of the cerebellar cortex

Mef2 family of TFs has been shown to carry out a diverse range of functions in brain development and maintenance (Lin et al., 1996; Potthoff and Olsen., 2007; Shalizi and Bonni., 2005). Previous findings show that collectively, *Mef2* gene homologues regulate the expression of several neuromodulatory factors such as *Arc* and *Bdnf* but the functions of each of these genes and whether they execute tasks independently or in tandem with each other are some of the questions that are yet to be answered (Flavell et al., 2006). While functional studies have revealed that *Mef2a* and *Mef2d* regulate neuronal survival, they have also been showed in other independent studies to control the formation of excitatory synapse numbers indicating they are capable of carrying out a variety of neuromodulatory events during development (Fiore et al., 2009; Shalizi et al., 2006).

While *Mef2a* and *Mef2d* are found in all cerebellar layers, *Mef2c* is restricted to the PL, indicating that *Mef2* TFs may carry out independent or overlapping tasks in the cerebellum (**Figure 4-2**). Furthermore, from our analysis, we also show that *Mef2a* and *Mef2d*, also expressed by the Purkinje cells have been reported to play roles in the differentiation of cortical

neurons during development (Flavell et al., 2006) (**Figure 4-2**). It is therefore possible that even though loss-of-function tests revealed that downregulation of *Mef2a* and *Mef2d* leads to the apoptosis of cerebellar granule cells (Li et al., 2001), they could also work with *Mef2c* in the Purkinje cells to regulate its proper differentiation and maturation. Further characterization of *Mef2a* and *Mef2d* across developmental stages and loss-of-function assays will be useful in verifying the possibilities of *Mef2* TF interactions.

5.2 *Mef2c* is an essential intrinsic regulator of postnatal Purkinje cell development

The dynamic molecular and morphological changes that Purkinje cells undergo in the first three weeks of postnatal development have been well documented in previous studies (Sotelo and Dusart., 2009). During this period Purkinje cells undergo migration, spinogenesis, synaptic reorganization and the growth and regression of dendrites, all in concert with each other (Dusart and Flament., 2012). A recent study knocking down transcription factor ROR α at selective time points revealed that it coordinates independent neurodevelopmental processes which occur at a given time in both developing and mature Purkinje cells (Takeo et al., 2015). Using a similar experimental design, we eliminated *Mef2c* expression at P3, a time point at which Purkinje cells have been reported to show increased susceptibility to cell death (Sotelo., 1985).

Upon analyzing the GFAP levels and relative soma size in control and knockdown groups, we learned that unlike other *Mef2* family members, *Mef2c* does not regulate neuronal viability (**Figure 4-5**). Also, the loss of *Mef2c* did not affect other aspects of Purkinje cell development such as elimination of somatic dendrites from P0-P4 and monolayer alignment from P0 to P3 (**Figures 4-6, 4-7**). These results indicate that although *Mef2c* might not regulate

these processes, it might have roles in other intrinsically-driven neurodevelopmental changes that occur in the first three postnatal weeks of Purkinje cell development.

Spinogenesis in Purkinje cells, controlled predominantly by intrinsic factors, begins at P3 which also corresponds to the age when functional CF synapses form onto Purkinje cells (Sotelo, 1985). Although the exact time point of initiation is not clearly defined, Purkinje cells also receive inputs from immature PFs and possibly direct but transient MF input within the first postnatal week (Miyazaki et al., 2003; Takeda and Maekawa., 1989). Classification of spines on Purkinje cells have revealed the presence of two types namely, the thorns which are found in the proximal regions and spiny branchlets which exhibit a more distal distribution (Sotelo., 1978). Thorns are mainly found on the primary and secondary dendrites and are the points of contact for CF-Purkinje cell synapses while spiny branchlets do the same but for PF-Purkinje cell synapses (Hasimoto et al., 2009).

There have been several reports of genetic regulators of Purkinje cell spinogenesis. For example, a knockdown of *RORα* showed a complete loss of spines on developing Purkinje cells (Takeo et al., 2015). In another study, the knockdown of the gene *Fmr1*, responsible for the fragile X syndrome, revealed the presence of elongated spines on Purkinje cells (Koekkoek et al, 2005). Contrary to both these studies, the knockdown of other genes such as *Disc1*, associated with ASD, has been reported to regulate spines of excitatory cortical neurons but not that of Purkinje cells (Hayashi-Takagi et al., 2010). Therefore, spinogenesis of the cerebellar Purkinje cells is regulated by a select few gene candidates and identifying *Mef2c* as one of these candidates will add to the list of potential therapeutic targets for neurological disorders showing morphological defects.

Purkinje cells start receive many excitatory inputs between P0 and P10, suggesting that an increase in spine numbers could be occurring to cater to this rapid increase in synaptogenesis (Miyazaki et al., 2003). Furthermore, a study that characterized the direct downstream targets of *Mef2* revealed that it regulates two sets of genes, one that works to strengthen excitatory synapses while the other weakens the same (Flavell et al., 2008). Interestingly, Purkinje cells also undergo simultaneous strengthening of PF-Purkinje cell synapses and weaken of excess CF-Purkinje cell synapses in the first and second weeks of Purkinje cell development (Hashimoto et al., 2009). Therefore, it is probable that *Mef2c* regulates spinogenesis by selectively regulating the expression of genes that concurrently strengthen and weaken excitatory synapses but this theory warrants more experimental evidence to be established.

5.3 Insights into *Mef2c*-modulated changes in Purkinje cell morphology during specific stages of cerebellar development

Purkinje cells undergo well-characterized changes in morphology in the first three postnatal weeks of cerebellar development, but the underlying mechanism of this process remain elusive (Takeo et al, 2015). While the processes that occur in the first week of Purkinje cell development are predominantly driven by intrinsic factors, the events that take place in the second and third postnatal weeks are regulated by the coordination of both intrinsic and external factors (Sotelo and Dusart., 2009). From our findings, we show that the loss of *Mef2c* in the first postnatal week shows an increase in total number of intersections in Purkinje cells at two independent ages, P14 and P21 suggesting that *Mef2c* intrinsically regulates dendritogenesis (**Figures 4-9-1, 4-9-2**). Additionally, knockdown studies at P7, continued to show an increase in dendritic intersections at P21, confirming that *Mef2c* is an essential intrinsic mediator of dendrite formation in developing Purkinje cells (**Figure 4-9-3**).

Previous findings, characterizing signaling cascades responsible for dendritic formation, identified a few calcium-mediated pathways that might have important roles to play in this process (Miller and Kaplan, 2003). Interestingly, some of the key upstream regulator in these cascades were CDK5, CAMKII and ERK (Miller and Kaplan, 2003), all three of which directly control the activation of *Mef2* TFs, suggesting that one or all of these cascades could control dendritogenesis of Purkinje cells (Miller and Kaplan., 2003; Shalizi and Bonni., 2005). Additionally, *Mef2* has been reported to execute its roles in dendritogenesis by employing downstream targets such as miRNAs and other translational modulators (Fiore et al., 2009). In conclusion, the experimental validation of these pathways, as summarized in **Figure 5-3**, will help us gain better insights of the workings of *Mef2c*-regulated control of dendrite formation in Purkinje cells and also in identifying potential therapeutic targets for Purkinje cell-related neurological disorders.

Figure 5-3: Summary of signaling pathways that might regulate morphogenesis of developing Purkinje cells in the cerebellum (Solid arrows= direct targets, dashed arrows= indirect targets)

5.4 Regulation of excitatory-inhibitory synaptogenesis by *Mef2c* in a stage-dependent manner

Mef2c has been previously reported to regulate both excitatory and inhibitory synapses in excitatory neurons during the developmental stages of the cerebral cortex and hippocampus (Harrington et al., 2016). GAD67 puncta at the soma of P14 Purkinje cells are derived from two sources. The first comes from neighbouring Purkinje cells, and the second from the stellate and basket cell population which have just completed proliferation and have initiated migration to the ML and start strengthening their inhibitory inputs on Purkinje cells, reported to be very potent at P14 (Witter et al., 2016; Pouzat and Hestrin., 1997). P14 is also the age at which excess CF elimination on Purkinje cell soma terminates, leaving very little excitation to balance the GABAergic inputs (Hashimoto and Kano., 2005). Since the process of CF-PF elimination has been reported to be controlled by intrinsic factors in an activity-dependent manner it is possible that a loss of *Mef2c* might have disrupted this process, explaining the increase in somatic GAD67 and also unchanged levels of vGluT2 in the soma (Mikuni et al., 2013). Similarly, in the dendrites at P14, if the CF-PC synapse is altered, we would expect very little mature excitatory inputs from CF when compared to the numerous GAD67 input from stellate and basket cells, explaining the presence of a higher number of inhibitory synapses (Pouzat and Hestrin., 1997; Hashimoto and Kano., 2005).

In contrast to P14, we observe a significant reduction in GAD67 in the soma and vGluT2 in the dendrites of P21 Purkinje cells after *Mef2c* knockdown. *Arc*, an activity-dependent regulator of CF-Purkinje cell elimination on Purkinje cell soma, was shown to accelerate CF

pruning when Purkinje cells were optogenetically stimulated over prolonged periods observed only till P14 (Mikuni et al, 2013). Coincidentally, *Arc* is also a direct downstream target of *Mef2* suggesting that a knockdown of *Mef2c* could also reduce the levels of *Arc*, thereby delaying the elimination of CFs on Purkinje cell somata as seen with the unchanged levels of vGluT2 in the soma at P14 and P21 (**Figure 4-10-1 and 4-10-2**; Flavell et al., 2008). After P14 however, it is possible that the CF elimination process recovers, starting with elimination of CF synapses in the dendrites which might explain the reduced levels of vGluT2 in the dendrites (**Figure 4-10-1**).

The reduction of GAD67 density on the soma could arise from a reduction of inhibitory synapses of the stellate cells on Purkinje cell soma as they migrate upwards from the IGL to occupy the outermost layers of the ML while the basket cells remain aligned closer to the PL (Zhang and Goldman., 1996). Additionally, a knockdown of *Mef2c* might also lead to the increase in excitatory synapses of granule cells in the IGL, which in turn excite Purkinje cell soma directly, retaining sufficient excitatory signals to disrupt proper inhibition (Guo et al., 2016). Therefore, *Mef2c* might alter synapse formation on Purkinje cells by altering the excitatory synaptic inputs which in turn might also disrupt inhibitory influences. The disruption of E/I balance in the brain has been linked to several neurodevelopmental disorder such as autism, a disorder in which there have also been reports of reduced numbers of Purkinje cells (Gao et al., 2015; Nelson et al., 2015). We propose that *Mef2c* serves as a genetic entry point to understand the contributions of the cerebellum in ASD and other disorders that have a disrupted balance of excitation/inhibition.

6. SUMMARY

We characterized the RNA expression patterns of all four *Mef2* TFs in the ML, PL and IGL of the cerebellar cortex (**Figure 4-2**). *Mef2c* RNA transcript, unlike *Mef2a* and *Mef2d* which are found in all layers, is restricted to the PL and analysis of *Mef2c* protein expression with known markers for specific cerebellar neuronal subtypes shows that *Mef2c* is expressed exclusively in Purkinje cells, but not other cerebellar neuronal subtypes (**Figure 4-3-1**). Co-staining with Zebrin, a marker for Purkinje cell subsets in the cerebellum, revealed that *Mef2c* is expressed by all Purkinje cells (**Figure 4-3-2**). Furthermore, a spatio-temporal analysis of *Mef2c* expression showed that the onset of *Mef2c* expression in the cerebellar cortex occurs postnatally (**Figure 4-3-3**). The expression of *Mef2c* in Purkinje cells from early developmental stages to maturity suggests that it might be involved in the development of Purkinje cells during this period (**Figure 4-3-3**).

In order to explore our hypothesis, we specifically and efficiently knocked down *Mef2c* expression in Purkinje cells using shRNAs with Purkinje cell-specific promoter (**Figure 4-4**; Sawada et al, 2010). Loss of *Mef2c* between P1 and P14 collectively revealed that it is not involved in Purkinje cell viability, monolayer alignment, and regression of somatic dendrites (**Figure 4-5, 4-6, 4-7**). However, we discovered a significant increase in spine number in Purkinje cells that lacked *Mef2c* (**Figure 4-8**). We also observed that silencing *Mef2c* expression at P1 resulted in a significant increase of dendritic intersections in developing Purkinje cells at P14 and P21 (**Figure 4-9-1, 4-9-2**). On the other hand, when Purkinje cells were transduced at P7 and analyzed at P21, no significant changes were observed in number and distribution of intersections suggesting that *Mef2c* has a critical window of influence during normal dendritogenesis in Purkinje cells (**Figure 4-9-3**).

Analysis of synaptic profiles of Purkinje cells revealed that the suppression of *Mef2c* expression from P1 to P14 showed an increase in the number of GABAergic synapses in both the somatic and dendritic compartments. (**Figure 4-10-1**). However, analysis of P21 Purkinje cells, also transduced at P1, showed a decrease in GAD67 puncta on the soma and vGluT2 puncta on the dendrites (**Figure 4-10-2**). Since P14 and P21 correspond to two important periods of Purkinje cell development where dynamic changes in excitatory and inhibitory synapses occur simultaneously, it is very possible that *Mef2c* regulates the formation of either one or both of these processes.

To study the activity-dependent expression of *Mef2c*, we first carried out a food deprivation task that has been reported in previous studies to increase locomotor and neuronal activities (Koubi HE, 1991). We first measured the spontaneous activity in deprived and not deprived mice which showed elevated levels of locomotor activity in the former. By counting the number of *cFos* positive nuclei in the hypothalamus we confirmed that neuronal activation also occurs simultaneously (**Figure 4-11-1**). Analysis of *cFos* and *Mef2c* levels in three independent regions of the cerebellum revealed elevated levels of both genes in all Purkinje cells within these regions of interest suggesting that in addition to a role of *Mef2c* during the development of Purkinje cells, *Mef2c* expression may be regulated in the cerebellum in an activity-dependent manner (**Figure 4-11-2**).

7. LIMITATIONS AND FUTURE WORK

There are some limitations in this project that need to be addressed.

- Although the knockdown of *Mef2c* resulted in an increase in dendritic and spine numbers of Purkinje cells, it remains unknown if an overexpression of *Mef2c* would show the opposite effect. Therefore, it would be useful to perform a Purkinje cell-specific overexpression of *Mef2c* to check the possibility that there is a reduction in dendrites as well as spines upon overexpression. Accompanying this experiment with a sequencing analysis would also help in identifying off-target effects if any.
- This thesis covers the possibility that there is impaired climbing fiber and inhibitory inputs from stellate and basket cells which was analyzed using vGluT2 and GAD67. However, we are yet to assess how excitatory inputs from parallel fibers are affected. This can be determined using a co-localization analysis of vGluT1 and GFP.
- For studies exploring the possible roles of activity-induced *Mef2c*, we have identified that food depriving mice leads to increased *Mef2c* and *cFos* expression. However, it is yet to find out how deprivation and increased locomotion separately induces *Mef2c* expression. It would therefore be useful to determine *Mef2c* expression in control mice for food deprivation and locomotion using independent experiments.

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