NEW INSIGHTS INTO THE DEVELOPMENT OF CEREBELLAR NUCLEI NEURONS IN MICE

By

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ABSTRACT

Development of the central nervous system begins with the specification of the neuroectoderm, delineation of the neural sheet into segments that will give rise to different brain compartment, and follows with the massive neurogenesis and neuronal migration. Cerebellum is a part of this complicated system with largest number of neurons. During cerebellar development, cerebellar nuclei neurons and Purkinje cells are the earliest born among the different neuronal subtypes. Purkinje cells are the sole output of the cerebellar cortex and they project to the cerebellar nuclei, which in turn represent the main output of the cerebellum. The cerebellum is generated from the rhombic lip and the ventricular zone. We used whole mount/section immunohistochemistry, mouse cerebellar and embryonic cultures, Dye Tracers and *In Situ* Hybridization to examine the origin of a new subset of cerebellar nuclei neurons from the mesencephalon during early cerebellar development. The isthmic organizer plays an active role in differentiating the mesencephalon (midbrain) from the rhombencephalon (hindbrain). Our results show that a subset of cerebellar nuclei neurons, which are immunopositive for α-synuclein (SNCA) and orthodenticle homeobox 2 (Otx2), originate from the mesencephalon and cross the isthmus toward the rostral end of the nuclear transitory zone. Double immunoflourscence of the SNCA with Otx2 or p75 neurotrophin receptor (p75ntr) indicates that these cells are derived from neural crest cells. We also showed that this population of neurons with nerve fibers terminates at the subpial surface of putative lobules VI/VII. The $SNCA^{+}/Otx2^{+}/p75^{+}$ cells, which divide the cerebellar primordium into rosterodorsal and caudoventral compartments, show increased cleaved caspase-3 activation to activate axonal guidance. These results strongly suggest that early cerebellar nuclei neurons originate from the mesencephalic neural crest population. Their presence in the nuclear transitory zone suggests

that maybe these neurons/fibers have a regulatory role as a signaling center, such as for axonal guidance and neuronal migration during early cerebellar development. To question the importance of the expression of SNCA during cerebellar development we find a mouse with ablation of *Snca* gene and study it for any abnormalities during embryonic and adult cerebellum. Our findings indicate that the mesencephalic derived cells are still there while expressing P75NTR and not SNCA and this ablation does not interrupt the cerebellar architecture development and zone and stripe pattern formation and shown normal cerebellar morphology in postnatal mouse cerebellum. To explore and trace the distribution and pattern of p75NTR expression we used postnatal cerebellum in normal and *Acp2* mutant mice with cerebellar defect. It was shown that P75NTR expressed in the same pattern as HSP25 zone and stripe phenotype (strongly present in Purkinje cells of the central and nodular zones) in adult cerebellum in both control and *Acp2* mutant mice. In this thesis, I provide data about existence of the third germinal zone, the mesencephalon, in the developing mouse after that the role of SNCA expression was questioned during mouse cerebellar development. Beside spatiotemporal expression of SNCA, p75NTR expression was studied.

DEDICATION

To my loved ones;

My mom and dad, for their endless love, selflessness and encouragement

My caring beloved husband and friend, Shayan, for all of his infinite love and support

& my kind sisters and brother, they brighten my whole life with joy, I am grateful to have all of them

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ABBREVIATIONS

Ascl1	Achaete-Scute Family BHLH Transcription Factor 1
Atoh1	Atonal bHLH transcription factor 1
AZ	Anterior zone
CNS	Central nervous system
CZ	Central zone
E	Embryonic day
En1,2	Engrailed homeobox 1,2
EGL	External granular layer
EGZ	External germinal zone
Fgf8	Fibroblast growth factor 8
HH	Hamburger-Hamilton stage
Hoxa2	Homeobox A2
IsO	Isthmus organizer
GABA	γ-aminobutyric acid
Gbx2	Gastrulation brain homeobox 2
GFP	Green fluorescent protein
GLHS	Gomez-Lopez-Hernandez Syndrome

Gli2	Gli family zinc finger 2
Gsx1	GS Homeobox 1
MHB	Midbrain-hindbrain boundary
NeuroD1	Neuronal Differentiation 1
Ngn2	Neurogenin 2
NTZ	Nuclear transitory zone
NZ	Nodular zone
Olig2	Oligodendrocyte transcription factor 2 (bHLH transcription factor)
Otx2	Orthodenticle homeobox 2
Pax6	Paired box protein 6
PCP	Purkinje cell precursors
Pf	primary fissure
PIPs	Pax2-positive interneuron progenitors
Plf	posterolaterl fissure
PNS	Peripheral nervous system
Ppf	prepyramidal fissure
Ptch1	Patched1
Ptf1a	Pancreas specific transcription factor 1a

PZ	Posterior zone
r1	rhombomere-1
RL	Rhombic lip
sc	secondary fissure
SHH	Sonic hedgehog
Smo	Smoothened
Tbr1	T-box, brain, 1
Tbr2	T-box, brain, 2
Slit	Slit Guidance Ligand
SVZ	Sub-ventricular zone
VZ	Ventricular zone
Wnt1	Wingless-type family member 1

CHAPTER ONE: INTRODUCTION

In this chapter, I will explain about the basic structure and function of the central nervous system and in particular, cerebellar structure, function, histology, afferent, efferent and development (neurogenesis, gliogenesis and neuronal migration). My main focus will be on development of cerebellar nuclei from neurogenesis and migration to final destination in nuclear transitory zone (NTZ). Despite their functional significance, our knowledge of cerebellar nuclei neurons development is incomplete. It is postulated that the developmental mechanisms responsible for cerebellar nuclei patterning are a key locus of evolutionary adaptation (Leto et al., 2016). Part of the literature review done in this chapter is derived from my previous publications which are addressed in the glossary, as followed:

1) Rahimi Balaei M, Ashtari N, Bergen H. The embryology and anatomy of the cerebellum. Development of the cerebellum from molecular aspects to diseases, 33-43.
2) Rahimi-Balaei M, Afsharinezhad P, Bailey K, Buchok M, Yeganeh B, Marzban H Embryonic stages in cerebellar afferent development. Cerebellum Ataxias. 2015 Jun 11;2:7.
3) Rahimi-Balaei M, Bergen H, Kong J, Marzban H. Neuronal migration during development of the cerebellum. Submitted to Frontiers in Cellular Neuroscience. 2018.

1.1 Overview of the central nervous system; structure

Our body is controlled by the nervous and endocrine systems. The nervous system is divided into two parts - central nervous system (CNS) and peripheral nervous system (PNS). CNS consists of the brain (encephalon) and spinal cord (medulla spinalis). The brain is located inside the skull, which provides mechanical support while the spinal cord lies inside the spinal canal (from foramen magna to second lumbar vertebrate) (Snell, 2010). The fully developed human brain weighs approximately 1400 g at early adulthood and contains several hundred billion of cells, including over 100 billion neurons (Dekaban and Sadowsky, 1978, Pakkenberg and Gundersen, 1997). Both the brain and spinal cord are covered with a system of membranes, called meninges (dura, arachnoid, and pia maters), and are suspended in the cerebrospinal fluid. Functionally, brain controls our awareness, motion, sensation, thought, speech, and memory. Besides, the spinal cord has its own movement reflexes independent of the brain. The nervous system is comprised of specialized cells called neurons (the long processes of a neurons are called axons or nerve fibers) to receive sensory stimuli, process in interneurons, and transferring suitable response to target organs (Snell, 2010).

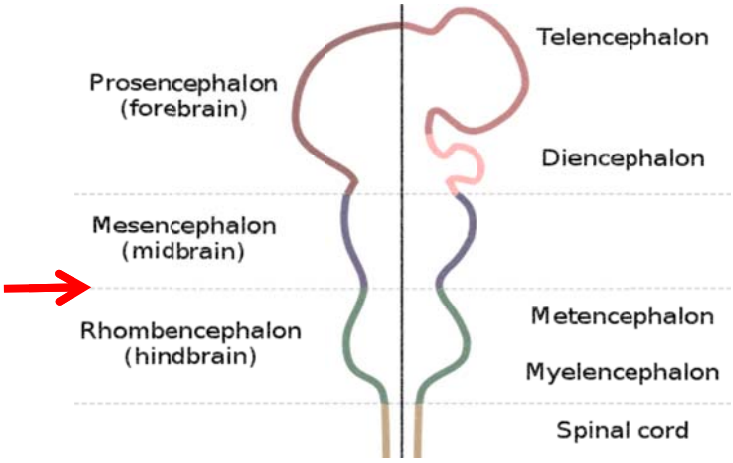

Figure 1. 1 Diagram depicting the main subdivisions of the embryonic vertebrate brain, adopted from https://en.wikipedia.org

Anatomically, brain is divided into the cerebrum, the cerebellum and brain stem and ontologically, it is divided to prosencephalon or forebrain, the mesencephalon or midbrain and the rhombencephalon or hindbrain (Snell, 2010, Sidman, 1982). The rhombencephalon consists of the medulla oblongata or myelencephalon and the metencephalon (figure 1.1). The rostral expansion of the spinal cord forms the medulla oblongata. The metencephalon is divided into ventral and dorsal portions, the large ventral swelling, known as the pons and dorsal expanded portion, which will be differentiated to the cerebellum. The isthmus is located at the border of hindbrain and midbrain, which is also the farthest rostral portion of the hindbrain. The mesencephalon located between the rhombencephalon and the prosencephalon. Together, the medulla oblongata, pons and mesencephalon form the brain stem. The prosencephalon comprises the diencephalon (between brains) and the telencephalon (endbrain). The nuclear component of diencephalon is the thalamus and the hypothalamus. The telencephalon is mainly composed of the two cerebral hemispheres which are the largest parts of the brain and surround the diencephalon and the midbrain. The surface of the cerebral hemispheres is characterized by a number of convolutions (gyri) which are separated by fissures (sulci). The formation of the longitudinal fissures result in the subdivision of the cerebral hemispheres into five distinct lobes with different functions: frontal, parietal, temporal, occipital and limbic (Sidman, 1982, Snell, 2010).

1.2 Overview of the central nervous system; development

In humans, the embryonic period begins from fertilization to the end of gestational week 8. By the end of this period, the basic structure of the brain has formed and will develop into major divisions of the CNS and peripheral nervous system. The third week of gestation is characterized by the occurrence of a process known as gastrulation in which an embryo with three layers (the endoderm, the mesoderm, and the ectoderm) is formed from a two-layered embryo accompanied by the formation of different cell lineages like the neural progenitor cells (Stiles and Jernigan, 2010). The embryonic period is followed by the fetal period, which extends into the second trimester. In this stage, the neocortex develops beside extensive neural cell proliferation, migration, differentiation and circuitry (Rakic, 2002). The central nervous system is derived from the neural plate that is a thickened and elongated paramedian zone ectoderm (germinal layer) in humans and other vertebrates. The neural plate continues growing and starts folding to form the neural fold. The primordial neural crest separates the primordial neural ectoderm from the primordial general body ectoderm. Gradually, the neural fold (groove) deepens and its edges fuse dorsally to form the neural tube (neurulation happens between week 3 and 4) in which the primordial neural walls surround a continuous fluid filled ventricular cavity (Copp et al., 2003). This cavity persists as a system of four communicating ventricles in the adult brain and narrow spinal central canal. The closure of the neural plate occurs along the forebrain and cervical spinal cord primordial and continue rostrally and caudally to complete this process (neurulation) (Snell, 2010). Between weeks 4 to 8, many neural progenitors migrate and differentiate and after the neural tube closure, a portion of neural crest cells aggregates to form the sensory ganglia of cranial and spinal nerves. The neural progenitors originate from the ventricular zone (VZ) as it is located where the ventricular system will develop. The neural progenitors also proliferate in and

migrate from a layer beyond the VZ, which is named the subventricular zone (SVZ). The neural tube divides to form the roof plate, floor plate and lateral plates. Also, the sulcus limitans divides the lateral wall of the neural tube into basal and alar plates. The ventricular cavity consists of the rhombencephalic fourth ventricle (diamond-shaped), diencephalic third ventricle (the narrow, slit-like) and the two large lateral ventricles in the cerebral hemispheres (crescent-shaped). The lateral ventricle communicates with the third ventricle via the interventricular foramen. The third and fourth ventricles are interconnected by the narrow, mesencephalic cerebral aqueduct (Snell, 2010, Stiles and Jernigan, 2010). Neurogenesis continues until late fetal period and gliogenesis starts prenatally and continues postnatally. The neuronal migration starts at early embryonic stage and continues postnatally and many use glial cells serving as guidance and substrates to migrate (Stiles and Jernigan, 2010).

1.3 Structure and function of the cerebellum

Aristotle first described the cerebellum in the fourth century BC. He detailed a structure located at the back of the brain that shared some similarities to the brain, but noted that it differed in form and size. He therefore coined this structure 'parencephalis' in Ancient Greek, the Latin equivalent of which can be translated literally as 'little brain', a phrase which is still used to this day when referring to the cerebellum. The cerebellum is a critical region in motor (controlling movement and balance) and non-motor (emotion, cognition and language) functions. All of these actions are the result of connections between the cerebellum and many cortical and subcortical structures in the cerebral hemispheres. The cerebellum performs as a modulator for many of the cognitive, languages, motor, sensory, and emotional functions associated with different cerebral regions. The cerebellum is a highly organized structure, which can be divided

into two longitudinal regions along the rostral to caudal axia. These regions are anatomically named the vermis and hemisphere (figure 1.2). Mediolaterally, the cerebellum is divided and folded by fissures to form three lobes- anterior, posterior and flocculonodular (rostrocaudally) (figure 1.3). The primary fissure (pf) separates the anterior and posterior lobes, while posterolateral fissure separates the posterior and the flocculonodular lobes. The cerebellar lobes are subdivided further into lobules (lobules I-X). In addition, the cerebellum is organized in four "transverse" zones from rostral to caudal which is based on the gene expression and afferent fiber termination - the anterior zone (corresponding approximately to lobules I–V in mice), the central zone (CZ: lobules VI– VII), which can be further subdivided into anterior (CZa) and posterior (CZp) components the posterior zone (PZ: lobules VIII–IX), and the nodular zone (NZ: lobules IX–X) (Marzban and Hawkes, 2011, Sillitoe et al., 2005, Ozol et al., 1999a).

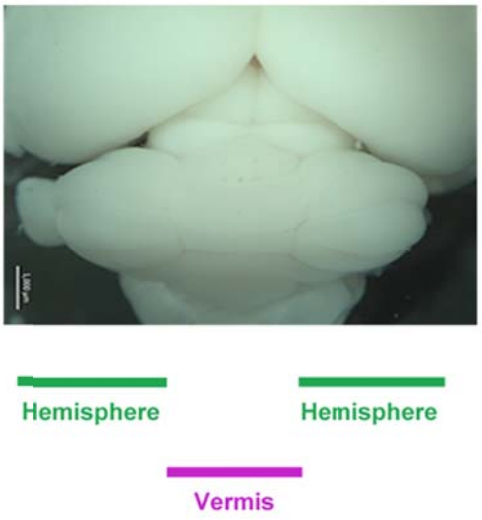

Figure 1. 2 External feature of the cerebellum from dorsal aspect in mice and its divisions: vermis and hemispheres

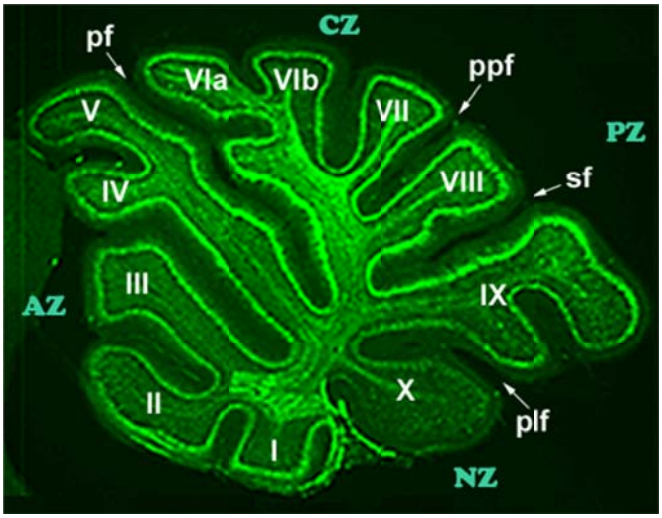

Figure 1. 3 Cerebellar fissures and zones in sagittal section of the mice cerebellum; AZ: lobules I–V, CZ: lobules VI– VII, PZ: lobules VIII–IX, NZ: lobules IX–X.

AZ: anterior zone, CZ: central zone, PZ: posterior zone, NZ: nodular zone, pf: primary fissure, ppf: prepyramidal fissure, sf: secondary fissure, plf: posterolateral fissure.

The cerebellum is attached to the midbrain, pons and medulla oblongata through three peduncles, which comprises the afferent to and/or efferent from the cerebellum. Afferent and efferent fibers are major components of cerebellar circuitry and the impairment of these circuits causes severe cerebellar malfunction, such as ataxia (Reeber et al., 2013, Beckinghausen and Sillitoe, 2019). The middle peduncle is the largest one and is attached to the pons, while the superior peduncle is connected to the midbrain and the inferior peduncle is attached to the medulla. Cerebellar afferents can be categorized into two major types: climbing fibers and mossy fibers, and a minor set of afferents projects to the cerebellum, which are called neuromodulatory fibers. Climbing fibers are generally derived from the inferior olivary complex and enter the cerebellum through the inferior cerebellar peduncle contralaterally, they synapse directly with

cerebellar nuclei or Purkinje cells of the cerebellar cortex (Desclin, 1974, Sugihara et al., 1996). Mossy fibers constitute the majority of afferent fibers in the adult cerebellum and arise from multiple sources. They communicate with cerebellar nuclei neurons and Purkinje cells through granule cells/parallel fibers. There are four main groups of mossy fibers that project to specific parts of the cerebellum (Rahimi-Balaei et al., 2015b):

1) The somatosensory projections (spinocerebellar and trigeminocerebellar fibers), which originate from the spinal cord and the trigeminal system (ganglion and nuclei) respectively, and project primarily to lobules I-VI and lobule VIII (Yaginuma and Matsushita, 1989, Steindler, 1985).

2) Vestibulocerebellar projections, which originate from the vestibular system (ganglion and nuclei) and terminate in ventral lobules IX and the flocculus with the adjacent ventral flocculonodular lobe (Voogd et al., 1991, Epema et al., 1990).

3) Reticulocerebellar projections, which originate from the lateral reticular nuclei, the paramedian reticular nucleus and the nucleus of Roller, and nucleus reticularis tegmenti pontis terminate throughout the cerebellum, but generally mirror the projections of the spino/trigeminocerebellar and vestibulocerebellar systems (Valle et al., 2001).

4) Pontocerebellar projections, which originate from the pontine nuclei and terminate in the cerebellum, particularly the vermis and hemispheres of lobules VI-VII (central zone), but not in the nodulus and flocculus (Serapide et al., 1994).

Neuromodulatory cerebellar afferent fibers terminate in all three layers of the cerebellar cortex (will be explained in detail in the histology of the cerebellum section) and the cerebellar nuclei to modulate other afferents (mossy and climbing fibers) and they are categorized

according to their neurotransmitters into serotonergic, noradrenergic, acetylcholinergic, dopaminergic, and histaminergic neuromodulatory afferents (Jaarsma et al., 1997, Schweighofer et al., 2004).

Functionally and phylogenetically (evolutionary), the cerebellum is divided into three divisions; vestibulocerebellum (archicerebellum), spinocerebellum (paleocerebellum) and cerebrocerebellum (neocerebellum) (Balaei et al., 2017, Haines and Dietrichs, 2012).

The spinocerebellum consists of the vermis and intermediate parts of the hemispheres referred to as the paravermis. It regulates body and limb movements (axial and proximal limb muscle musculature) and its inputs are largely mossy fibers originating from the spinocerebellar tract (dorsal ascending tracts) and cuneocerebellar tract. The spinocerebellum then projects to the interposed nucleus (paravermal part) and fastigial nucleus (vermal part).The efferent of spinocerebellum project to cerebral cortex after relay in thalamus (Balaei et al., 2017, Haines and Dietrichs, 2012).

The vestibulocerebellum (flocculonodular lobe) is the oldest part of the cerebellum phylogenetically and consists of the midline nodulus and the bilateral floccule. It receives input (mossy fibers) from the vestibular ganglion (sensory information about motion, equilibrium, and spatial orientation in each ear) and vestibular nucleus in the brainstem. It regulates balance and eye movements, so it receives additional visual input from superior colliculi. The fastigial nucleus is the principal of the vestibulocerebellum and project excitatory bilateral projections to the vestibular nuclei (inferior cerebellar peduncle). In addition to the fastigial nucleus, the cortex of the vestibulocerebellum projects directly to the vestibular nuclei of the brainstem to coordinate the vestibular ocular reflex. Then, fibers from vestibular nuclei project to the spinal cord, to form the vestibulospinal tract. This tract maintains balance by activating anti-gravity

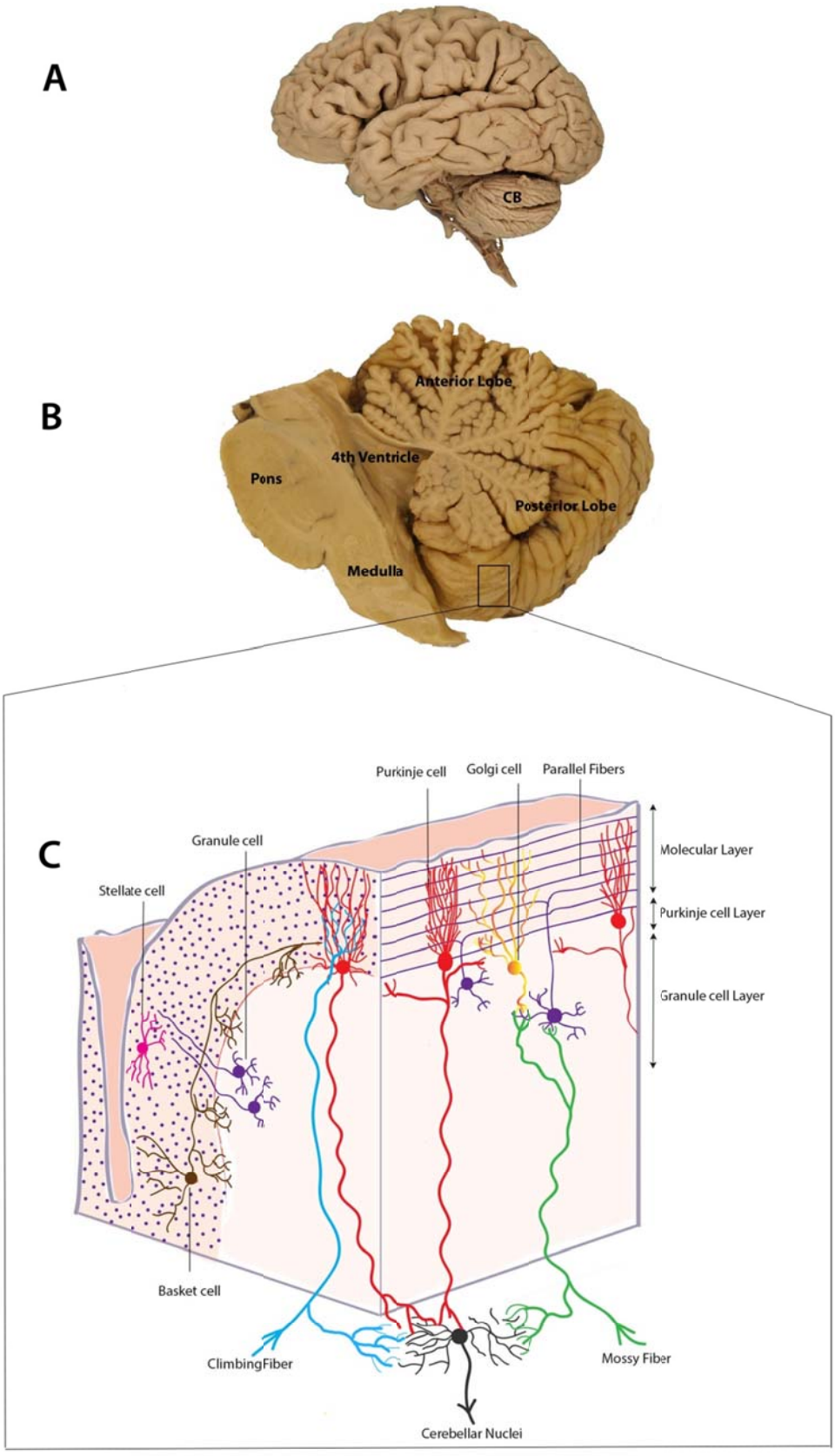
A
CB
B
Anterior Lobe
4th Ventricle
Pons
Posterior Lobe
Medulla
C
Purkinje cell
Golgi cell
Parallel Fibers
Granule cell
Stellate cell
Molecular Layer
Purkinje cell Layer
Granule cell Layer
Basket cell
ClimbingFiber
Mossy Fiber
Cerebellar Nuclei

Figure 1. 4 The anatomy and histology of the cerebellum

(**A**) Location of the cerebellum in situ. (**B**) Hemisected view of the cerebellum showing the vermis, the locations of the anterior and posterior lobes, and its anatomical relationship to the brainstem. (**C**) Schematic representation of the cerebellum showing the mossy fiber and climbing fiber inputs to the cerebellar cortex. The mossy fibers contact the granule cells and send collaterals to the cerebellar nuclei while the climbing fibers make contact with the dendrites of the Purkinje cells and may also send projections to the cerebellar nuclei. The granule cells project to the molecular layer and bifurcate to form the parallel fibers that contact the Purkinje cell dendrites as well as the basket cells and stellate cells. The Golgi cells receive input from mossy fibres and also project into the molecular layer of the cortex. The image is from (Balaei et al., 2017).

muscles of the lower body. Ascending projections from fastigial nucleus (superior cerebellar peduncle) project to thalamus and relay to corticospinal neurons to involve in balance and posture musculature (Balaei et al., 2017, Haines and Dietrichs, 2012).

The cerebrocerebellum or pontocerebellum is the largest functional component of the cerebellum. It consists of the lateral parts of the hemispheres and is involved in planning movement and evaluating sensory information for action. It receives input from cerebral cortex (frontal and parietal) contralaterally (Balaei et al., 2017, Haines and Dietrichs, 2012).

The cerebellar nuclei and the lateral vestibular nucleus constitute the sole output (efferent) of the cerebellum, and play a central role in cerebellar circuitry and function. Purkinje cell axons are the sole output of the cerebellar cortex and integrate all cerebellar inputs before projecting to the core of the cerebellum to form inhibitory synapses on their target cerebellar nuclei neurons. The

cerebellar nuclei are the final efferent pathway to the rest of the brain and spinal cord; however, a small minority of Purkinje cells project directly to vestibular nuclei. The superior cerebellar peduncle consists primarily of efferent fibers from the dentate and interposed nuclei projecting to the contralateral red nucleus and ventral lateral nucleus of the thalamus. The cerebellar efferents of the spinocerebellum that project to nuclei of the reticular formation also pass through this peduncle (Balaei et al., 2017, Haines and Dietrichs, 2012).

1.4 Histology of the cerebellum

Several cell types are aggregated in the cerebellum to form the cerebellar gray matter, which includes the cerebellar cortex and the cerebellar nuclei (figure 1.4). The white matter is present below the cerebellar cortex and around cerebellar nuclei. The cerebellar cortex contains three different layers, which are, from the outside to inside, the molecular layer, the Purkinje cell layer, and the granular layer. The three-layered cerebellar cortex contains six main neuronal cell types: stellate and basket cells in the molecular layer, Purkinje cell somata in the Purkinje cell layer (along with the somata of Bergmann glia and candelabrum cells), and granule cells, Golgi cells, and unipolar brush cells in the granular layer (plus Lugaro cells) (figure 1.4 and figure 1.5). The two most distinctive cells in the cerebellar cortex are the large Purkinje cells and the small granule cells. The granular layer is densely packed with granule cells, small neurons whose axons extend into the molecular layer. Purkinje cells are the principal neurons of the cerebellar cortex, and the sole output of cerebellar cortex projects an axon to the cerebellar nuclei. The Purkinje cell layer consists of a single row of Purkinje cell soma, which are large neurons with a single axon-extending deep into the cerebellum (cerebellar nuclei) and multiple dendrites branching extensively in the molecular layer. The molecular layer has a low cellular density of

stellate and basket cells and contains mostly the axons of granule cells and the dendrites of Purkinje cells. Beneath the cerebellar cortex is the cerebellar white matter. Neurons of the cerebellar nuclei are located close to the roof of the fourth ventricle deep within the cerebellar white matter. In humans, paired cerebellar nuclei are arranged from lateral to medial dentate nuclei in the hemispheres, emboliform and globose (interposed nuclei) in the paravermis (intermediate), and fastigial nucleus in vermis. The cerebellar nuclei receive input from Purkinje cells (inhibitory, GABAergic) in the cerebellar cortex and mossy fiber and climbing fiber pathways (excitatory, glutamatergic) (Marzban, 2017, Sillitoe, 2016).

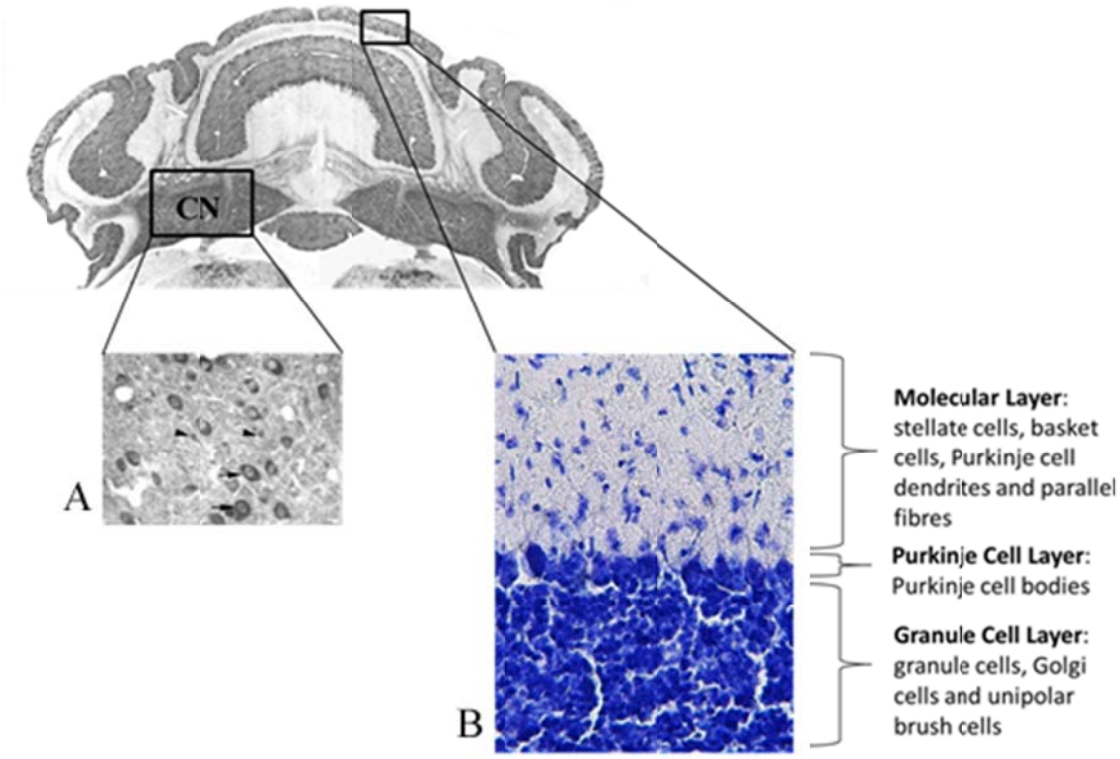

Figure 1. 5 Transverse sections of the adult mouse cerebellum shown cerebellar nuclei (A) and cortex (B)

(A) Arrows show large glutamatergic cerebellar nuclei neurons, arrowheads show small GABAergic cerebellar nuclei neurons

(B) Different layers of cerebellar cortex with their cellular content

1.5 The development of the cerebellum

1.5.1 Cerebellar primordium and isthmus organizer

In mice, the cerebellar primordium appears around embryonic day (E) 7-8in the neuroepithelial layer of the alar plate in rhombomere-1 (the rhombencephalon is subdivided to eleven rhombomeres (Tomás-Roca et al., 2016)). The cerebellar primordium is rostrally limited to the isthmus, a narrowing center at junction of the mesencephalon-rhombencephalon (rhombomere-1, r1) or midbrain-hindbrain boundary (MHB). Orthodenticle homeobox 2 (Otx2) and gastrulation brain homeobox 2 (Gbx2) are among the earliest genes expressed in the neuroectoderm, dividing it into anterior and posterior domains with a common border that marks the mid-hindbrain junction (the pattern of the expression of these genes meet at the mid-hindbrain border and form decreasing gradients in the opposite directions). Otx2 is required for development of the forebrain and midbrain, and Gbx2 for the anterior hindbrain. Otx2 expression ends rostral to the MHB in Hamburger-Hamilton stage (HH) 10 chick and in embryonic day (E) 9.5 in mice (Millet et al., 1996). By using chick-quail chimeras, it was shown that grafted Otx2-positive mesencephalic vesicles of the HH10 only give rise to midbrain primordium of the HH20 in the host. In contrary, they have shown that grafted Otx2-negative mesencephalic and rhombencephalic vesicles give rise to the cerebellar primordium of the HH20 (rhombomere-1) in the host. This questioned the existence of the boundary between the midbrain and the hindbrain resulting in the replacement of this term with "isthmus organizer (IsO)"

signaling center (Millet et al., 1996, Hallonet et al., 1990). There is a growing body of literature that confirms the Gbx2-positive, Otx2- and Homeobox A2 (Hoxa2, may be involved in the placement of hindbrain segments in the proper location along the anterior-posterior axis during development) -negative rhombomere 1 give rise to the cerebellum (Zervas et al., 2004, Wingate and Hatten, 1999, Wassarman et al., 1997, Hallonet et al., 1990). The mesencephalon and rhombomere-1 show different morphology, expressing an array of different genes to control their development (Wurst and Bally-Cuif, 2001). In addition to Otx2 and Gbx2 transcription factors which are expressed widely in the anterior and posterior embryonic brain domains, the secreted molecule fibroblast growth factor 8 (Fgf8) is induced within the anterior Gbx2-positive domain and becomes refined to the isthmus constriction that separates the mesencephalon and rhombomere-1 (Joyner et al., 2000a). Wingless-Type Family Member 1 (Wnt1) is induced broadly within the presumptive Otx2-positive mesencephalon domain and becomes tightly restricted to a ring at the posterior mesencephalon placed side by side to Fgf8 (Joyner et al., 2000a). Wnt1 is expressed in mesencephalon and Fgf8 in cerebellar primordium in mice at E8, and after that restricted to a band around MHB (Joyner et al., 2000a). Deletion of Wnt1 and Fgf8 also result in the loss of the midbrain and the cerebellum (McMahon and Bradley, 1990, Meyers et al., 1998). In contrast to Otx2+/Wnt1+ domain in the mesencephalon and Gbx2+/ Fgf8+ domain in rhombomere-1, the En1/2 genes (Engrailed Homeobox 1/2) are induced in a region that encompasses both the mesencephalon and rhombomere-1 (figure 1.6) (Wurst and Bally-Cuif, 2001). Furthermore, it is indicated that Fgf8 soaked beads were able to induce ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression at the diencephalon in chick. It is suggested that the re-patterning of the neural tube happens by introducing Fgf8b as the organizing molecule in the isthmus organizer and Wnt1 is required for

the maintenance of Fgf8 expression at the IsO (Martinez et al., 1999). On the other hand, it is indicated that Fgf8 normally elevates cell survival in MHB region while Wnt1 is found to elevate cell proliferation in the midbrain region (Panhuysen et al., 2004). Also, early fate mapping studies done in chick indicated that posterior mesencephalon is the origin of the mediorostral cerebellum while lateral and caudal cerebellum is derived from rhombomere-1 (Hallonet et al., 1990, Hallonet and Le Douarin, 1993). This claim was refuted as explained before; the cerebellum is not derived from the mesencephalon as defined by the posterior limit of Otx2 expression, which is initially anterior to the isthmic constriction in chick (Millet et al., 1996).

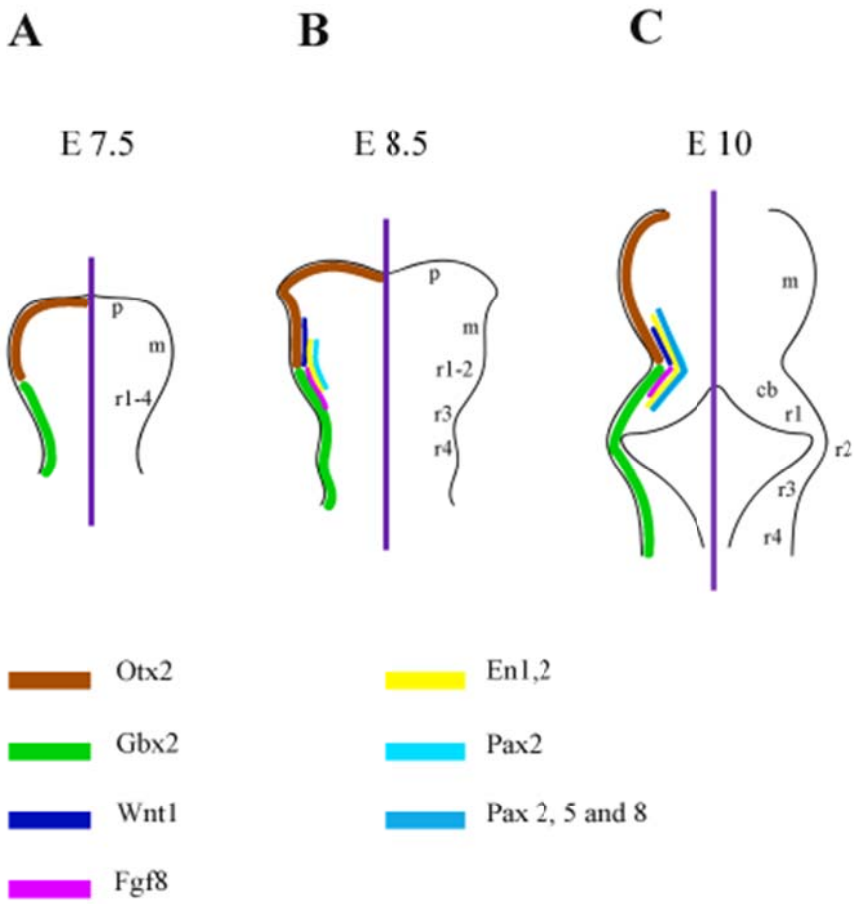

Figure 1. 6 Gene expression patterns at isthmus organizing center

Dorsal views of the mouse embryonic neural plate at **a** | the 0-somite stage, **b** | 6-somite stage and **c** | E10 stage, anterior to the top. **a** | 0 somite is at the end of gastrulation when the neural plate is subdivided into an anterior and posterior domains. Anterior domain expresses *Otx2* and posterior domain expresses *Gbx2*. **b** | At 6 somites, the border of *Otx2* and *Gbx2* expression domain touch each other. The expression of *Wnt1* is initiated in the mesencephalon, and *En1* (quickly followed by *En2*) and *Pax2* express in the *Otx2–Gbx2* border. Then, *Fgf8* expression starts in the caudal part of the *Otx2 –Gbx2* border. **c** | At E10, the *Otx2–Gbx2* border identifies the isthmus organizer or midbrain/hindbrain boundary. The expression of *Wnt1* and *Fgf8* has become restricted to narrow rings encircling the neural tube on either side of this boundary. The domains of *En1*and *Pax2* expression, which still overlap the boundary, have also become narrower, whereas *En2*, *Pax5* and *Pax8* are expressed across most of the isthmus organizing center. (*En*, engrailed; *Fgf8*, fibroblast growth factor 8; *Gbx2*, gastrulation brain homeobox 2; m, mesencephalon; cb, cerebellum; *Otx2*, orthodenticle homologue 2; P, prosencephalon; Pax, paired box; r, rhombomeres; violet line, axis of symmetry). This image is adopted from (Wurst and Bally-Cuif, 2001).

Around E7-8 the cerebellar primordium (anlage) appears at the neuroepithelium of the alar plate of rhombomere-1 in mice resulting in the division of rhombencephalic alar plate lips into two rostral and caudal rhombic lips. The cerebellum originates from the dorsal portion of the first rhombomere bilaterally including the rostral rhombic lip. The cerebellar primordium, which is the bilateral swallowing and connected with a thin midline portion, rotates from rostro-caudal to medio-lateral position as long as the midline bulk increases during development of cerebellum (Sillitoe and Joyner, 2007, Sgaier et al., 2005). The cerebellar nuclei and Purkinje cells are the

only neuronal populations that present during early cerebellar development, as the interneurons are born later.

1.5.2 Cerebellar germinal zones

The neurons that reside within the developing cerebellum are derived from two distinct germinal zones (a proliferative layer of the neural tube neuroepithelium): the ventricular zone (VZ) and the rhombic lip (RL), which produce different cerebellar neuronal types from E9 in mice (Sidman and Rakic, 1973).

1.5.2.1 Rhombic lip derived cells

The cerebellar neurons derived from the rhombic lip at the dorsal edge of the cerebellar primordium include the large neurons of the cerebellar nuclei (cerebellar output), unipolar brush cells (UBC), and the granule cells (the most numerous cell in the brain). All of these neurons originate from Atonal homolog 1 (Atoh1, atonal basic helix-loop-helix (bHLH) transcription factor 1, formerly known as Math1)-expressing neuronal progenitors) and use glutamate as their neurotransmitter (cerebellar glutamatergic neurons). Atoh1 expression is required for glutamatergic identity (Wang et al., 2005, Machold and Fishell, 2005). A previous study on *Atoh1* knockout mice revealed that the granule cells of external germinal (EGZ), glutamatergic cerebellar nuclei neurons and unipolar brush cells were missed, in addition to the This finding is against the earlier belief claiming that only granule cells have the rhombic lip origin. (Machold and Fishell, 2005, Ben-Arie et al., 1997a, Wang et al., 2005, Fink et al., 2006c, Englund et al., 2006a). Using *Ptf1a*^{Atoh1} knock-in transgenic mice whose ventricular zone produces glutamatergic cerebellar progenitors by ectopic expression of Atoh1, Yamada *et al*. showed the

role of Atoh1 expression in the production of glutamatergic neurons in the cerebellum (Yamada et al., 2014).

Fate mapping studies found the first neurons derived from the rhombic lip at E9-E12 were glutamatergic cerebellar nuclei neurons while granule cells are generated at E12.5-E17 (Machold and Fishell, 2005, Fink et al., 2006c, Sidman and Rakic, 1973). In addition, unipolar brush cells arise from rhombic lip at E15.5-17.5 in mice cerebellum(Englund et al., 2006a) (figure 1.7).

Cerebellar nuclei neurons

There are few studies about neurogenesis of cerebellar nuclei neurons in comparison to Purkinje cells and granule cells. At E10, Atoh1 expressing progenitors of cerebellar nuclei from rhombic lip migrate tangentially in the rostral migratory stream while expressing Pax6 and then subsequently expressing Tbr2. Following a change in direction and entry into the nuclear transitory zone (NTZ), the post mitotic cerebellar nuclei neurons express Tbr1 and aggregate in NTZ at E10-13. Further subdivision of the nuclei (lateral, interposed and medial) will be developed postnatally (Fink et al., 2006c). The expression of Tbr1 is maintained in medial (fastigial) nuclei into adulthood (in lateral and interposed nuclei continue until E14.5) which become disorganized in *Tbr1* null mice , The role of *Tbr2* could not be identified because of early embryonic lethality of *Tbr2* knockout mice (Ueno et al., 2000, Russ et al., 2000). In *Pax6* null mice, only the size of the cerebellum was decreased with lower foliation (Engelkamp et al., 1999b). Postmitotic precursors of the cerebellar nuclei neurons express the transcription factors Lhx2/9, Meis 1/2, Irx3 (Morales and Hatten, 2006) and Lmx1a (Chizhikov et al., 2010b).

Granule cell

Progenitors of granule cells (Atoh1 positive) originate from rhombic lip at E12.5-17. These cells migrate tangentially in the rostral migratory stream to form external germinal zone (EGZ), which is a layer of granule cell progenitors continue proliferation from E15 to P15. The proliferation of granule cell progenitors is under the influence of sonic hedgehog (Shh), which is secreted by the Purkinje cells at E17.5 and postnatally (Machold and Fishell, 2005, Espinosa and Luo, 2008, Dahmane and Ruiz-i-Altaba, 1999). Simultaneously, granule cell progenitors co-express Pax6, Meis1, Zic1/2 and Barhl1 (Morales and Hatten, 2006), but post mitotic granule cells stop Atoh1 expression (Akazawa et al., 1995). Using Bergmann glia fibers, granule cells in the inner layers of the external germinal zone start radial migration to form granular layer, and express NeuroD1 (early marker of the differentiated granule cells), Unc5h3 and Pax6 (Komuro and Yacubova, 2003b, Stoykova and Gruss, 1994, Miyata et al., 1999, Ackerman et al., 1997). Granule cells in the cerebellar anterior zone are born earlier than posterior zone granule cells (Machold and Fishell, 2005). Granule cell progenitors recognize Shh by Patched1 (Ptch1) receptors. Once activated, these cells release f Smoothened (Smo), which activates Shh pathway through Gli transcription factors (Gli2 is the main effector, Gli family zinc finger 2) (Dahmane and Ruiz-i-Altaba, 1999, Lewis et al., 2004, Flora et al., 2009). Using Atoh1 null mice, Flora *et al*. has demonstrated the role of Atoh1expression in Shh pathway and granule cell progenitors proliferation (Flora et al., 2009). Authors of this paper revealed that external germinal zone is not formed in Atoh null mice and the cerebellar size is reduced (Ben-Arie et al., 1997a, Machold and Fishell, 2005). Another study showed that Pax6 knockout mice exhibit developmental abnormalities such as small eye phenotype with small vermis, decreased foliation and thickened external germinal zone in hemispheres, axon guidance defects, cortical plate hypocellularity and

decreased basal ganglia volume. In humans, PAX6 haploinsufficiency is associated with the absence or hypoplasia of the anterior commissure and reduced olfaction (Engelkamp et al., 1999b, Sisodiya et al., 2001).

Unipolar brush cell

Previous research showed that progenitors of unipolar brush cells, which are originated from the rhombic lip, are born at E15.5-17.5 and express Atoh1, Pax6, Tbr2 and Lmx1a (Englund et al., 2006a). During cerebellar development, post-mitotic unipolar brush cells reduce the expression of Atoh1 within the rhombic lip, and start spreading into the core of the cerebellum at E18.5-P0 (Englund et al., 2006a).

1.5.2.2 Ventricular zone derived cells

The ventricular zone is the neuroepithelium of the alar plate of the rhombomere 1 that forms the roof or dorsal edge of the fourth ventricle. The neurons derived from the ventricular zone are Purkinje cells, Golgi cells, stellate cells, basket cells, and cerebellar nuclei interneurons. All of these neurons are derived from neural progenitors that express pancreas specific transcription factor 1a (Ptf1a) (bHLH transcription factor) and aregamma-aminobutyric acid (GABA) positive suggesting Ptf1a is vital for GABAergic identity of these cells (Hoshino et al., 2005). Also, these progenitors express Ascl1 (Achaete-Scute family bHLH transcription factor 1), which plays an important role in neurogenesis. Fate mapping studies showed that the neurogenesis take place in waves from ventricular zone at molecularly distinct sub-territories, which each of them gives rise to different cell types (Kim et al., 2008, Sudarov et al., 2011). In the first wave of neurogenesis, GABAergic cerebellar neurons appear at E10, while Purkinje cells generation is almost at the same time (E10) and ends at E13.5 (Kim et al., 2008, Hashimoto and Mikoshiba, 2003). The second wave of neurogenesis is when inhibitory interneurons (Golgi

cells, stellate and basket cells) are generated at E13.5-P7 (Leto et al., 2006). In addition, Seto *et al*. revealed that there are two subpopulations of dorsally and ventrally located progenitors, which are Olig2 (Oligodendrocyte transcription factor 2 (bHLH transcription factor)) and Gsx1 (genomic screen homeobox 1 transcription factor) positive within the Ptf1a domain at early stages (Seto et al., 2014b). GABAergic cerebellar neuron progenitors give rise to Purkinje cell precursors (PCP) and Pax2-positive interneuron progenitors (PIPs). The identity of GABAergic cerebellar neuron progenitors is temporally under the negative regulation of Olig2 and positive regulation of Gsx1 (Seto et al., 2014b). A research by Yamada and colleagues showed that GABAergic neurons are generated in rhombic lip with the ectopic expression of Ptf1a in *Atoh1*^{Ptf1a} transgenic knock-in mice (Yamada et al., 2014). A human study showed mutations in PTF1A cause pancreatic and cerebellar agenesis (Sellick et al., 2004). However, Pascual *et al*. showed that absence of Ptf1a not only resulted in cerebellar agenesis, but leads to abnormal differentiation of cerebellar GABAergic progenitors to an external granule cell-like phenotype (Pascual et al., 2007) (figure 1.7).

Purkinje cells

At E10-13, GABAergic Purkinje cells originate from ventricular zone-derived progenitors, which express Ptf1a (Hoshino et al., 2005, Sidman and Rakic, 1973). Besides Ptf1a, several other transcription factors such as Mash1 (or Ascl1), Ngn1 (Neurogenin 1), Ngn2 (Neurogenin 2), NeuroD1 (Neuronal Differentiation 1), Gsx1 and Olig1, 2 are expressed with distinct micro-domains in the cerebellar ventricular zone (Kim et al., 2008, Lundell et al., 2009, Florio et al., 2012, Seto et al., 2014b, Seto et al., 2014a, Zordan et al., 2008). As explained before, Olig2 counteracts with Gsx1 in the early cerebellar ventricular zone to maintain Purkinje cell progenitor identity (Seto et al., 2014b). In addition, it has been shown that Olig2 regulates

Purkinje cell generation in the early developing mouse cerebellum (Ju et al., 2016, Seto et al., 2014b). The deletion of Olig1 and Olig2 leads to a decrease in the Purkinje cell neurogenesis and increase in Pax2 positive interneurons (Seto et al., 2014b). Interestingly, when Olig2 (and not Olig1) is deleted, no obvious phenotypic defects occur. (Seto et al., 2014b, Ju et al., 2016). In addition, Ju *et al*., showed that during Purkinje cell generation, Olig2 is transiently expressed in the cerebellar ventricular zone, cerebellar ventricular zone progenitors and early-born neurons (Ju et al., 2016).

Although different subtypes of Purkinje cells are present in both medio/lateral positions in the adult cerebellum, but they have different origins in the ventricular zone. In this context, Purkinje cells located in the putative vermis (the most medial part of cerebellar primordium) are originated from isthmic rhombomere- derived ventricular zone, and Purkinje cells located in the hemispheres are originated from lateral parts of the ventricular zone (Altman and Bayer, 1985c). During cerebellar development, Purkinje cells develop earlier than granule cells and release Shh that is essential for the granule cell precursor proliferation. Under intricate regulatory system, a proper number and position of both cells are required to synapse and form cerebellar cortex circuitry (Lewis et al., 2004).

GABAergic interneurons (stellate/basket, and Golgi cells)

GABAergic interneurons originate from Ptf1a^{+} ventricular zone progenitors at E13.5-P7 (Leto et al., 2006, Leto et al., 2012). As mentioned before, the Ptf1a positive domain contains progenitors for two distinct cells; 1) Olig2 positive Purkinje cell progenitors, which are located more caudally and undergo their terminal mitosis between E11-13 and, 2) Gsx1 positive progenitors, which are located more rostrally and medially in the ventricular zone. In addition, at E12.5, Pax2 expressing interneuron precursors are in the rostral/medial microdomain (Leto et al.,

2006, Leto et al., 2012). In comparison to Purkinje cells, which migrate post-mitotically, Gsx1/Pax2 positive proliferating interneuron progenitors migrate in successive waves, with an inside-out progression, to the granular and molecular layers of the cerebellar cortex (Leto et al., 2006, Leto et al., 2012). Ju *et al*. argued and opposed the "temporal identity transition" introduced by Seto *et al*. and stated that the majority of Pax2 positive interneuron progenitors are transitioned from Olig2 positive Purkinje cell progenitors (Seto et al., 2014b) and they showed cerebellar Olig2 gene-expressing progenitors produce Purkinje cells, but not Pax2 positive interneurons in the developing cerebellum (Ju et al., 2016).

Cerebellar nuclei interneurons

At E12, the first wave of neurogenesis at ventricular zone is initiated and GABAergic cerebellar nuclei neurons are generated until E17. Ventricular zone progenitors require the expression of Ptf1a for GABAergic specification (Altman and Bayer, 1985c). As explained before, like other GABAergic interneurons, they are positive for Gsx1 and Pax2 and migrate in successive waves to the cerebellar nuclei (Leto et al., 2006, Leto et al., 2012). It has been shown that the GABAergic projection nucleo-olivary neurons are originated from ventricular zone lineage (Sudarov et al., 2011). In contrast to later-born GABAergic interneurons (Stellate/basket, and Golgi cells), both Purkinje cells and GABAergic cerebellar nuclei neurons express Neurog2 (Florio et al., 2012). Postmitotic cells expressing Neurog1 appear to be candidate cerebellar nuclei nucleo-olivary projection neurons (Zordan et al., 2008).

Cerebellar gliogenesis

During late embryonic and early postnatal development of the cerebellum, glial progenitors proliferate and differentiate into the astroglial cells, including the Bergmann glia (Altman and Bayer, 1997). Previous research by Fujita has demonstrated that gliogenesis in the

cerebellar cortex occurs postnatally (Fujita, 1967) while another study by Das showed the intensely labeled glial elements in cerebellar nuclei and white matter at embryonic period (Das, 1977). Gliogenesis is under the control of Notch1, and the ablation of Notch1 results in premature neurogenesis from neuroepithelium, premature gliogenesis with undifferentiated neurons and glial cells.

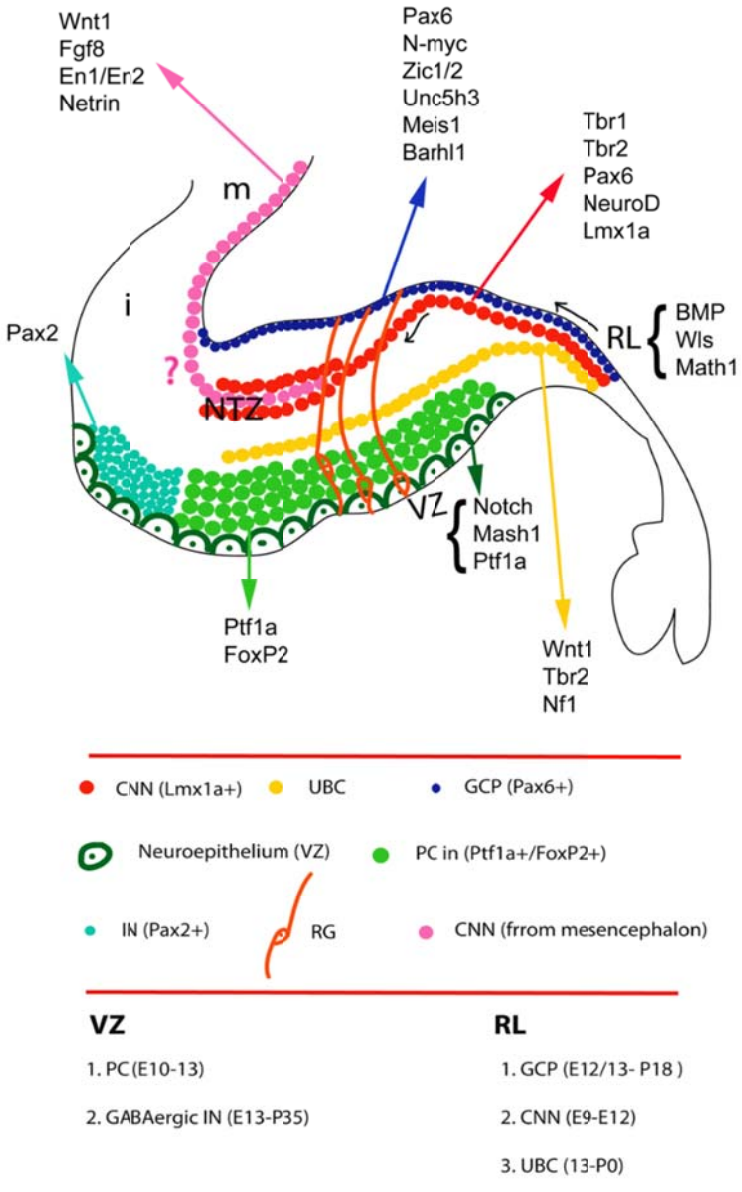

Figure 1. 7 Embryonic cerebellar neurogenesis and development

A schematic illustration of cerebellar development at sagittal section shows germinal zones in embryonic mice. Granule cell precursors, cerebellar nuclei neurons and unipolar brush cells originate at rhombic lip. Granule cell precursors (E12/13- P18) with cerebellar nuclei neurons (E9-E12) migrate in the rostral migratory stream. Purkinje cells (E10-13) and interneurons (Pax2+, E13-P35) originate from ventricular zone and Purkinje cells (Ptf1a+/FoxP2+) use radial glia as their substrate. m: mesencephalon, i: isthmus, NTZ: nuclear transitory zone, RL: rhombic lip, VZ: ventricular zone (neuroepithelium), GCP: granule cell precursor, UBC: unipolar brush cell, CNN: cerebellar nuclei neuron, PC: Purkinje cell, RG: radial glia, IN: interneuron, E: embryonic day, m: mesencephalon, i: isthmus.

1.6 Neuronal migration in developing cerebellum

Similar to the other regions of the brain, neuronal migration plays a substantial role in the development of cerebellar circuits (Hoshino et al., 2005). Abnormalneuronal migration is associated with a variety of brain disorders such as autism, ataxia and epilepsy (Gleeson and Walsh, 2000, Qin et al., 2017, Marzban et al., 2015, Kahler et al., 2008). Considering the simple cytoarchitecture of the cerebellum, with few neuronal types, there is an elaborate sequence of events, in which neurons that originate from different germinal zones migrate via complex migratory pathways to their final destination to assemble in a well-defined neuronal organization in the cerebellar cortex and nuclei (figure 1.8) (Rahimi-Balaei et al., 2018a) .

Neuronal migration follows either a radial or a tangential migratory pathway, depending on the area of the developing nervous system, in which the neurons originate. In radial migration,

nascent neurons follow a track that is perpendicular to the neuroepithelial surface and proceed alongside radial glial fibers (Nadarajah et al., 2001). In contrast, the tangential migration of neurons is parallel to the pial surface (Marín and Rubenstein, 2001).

1.6.1 Rhombic lip derived cells

Cerebellar nuclei neurons

Cerebellar nuclei neurons have a dual origin from the ventricular zone and the rhombic lip. The cerebellar nuclei comprise several neuronal types; large glutamatergic projection neurons, inhibitory GABAergic neurons (terminate in the inferior olive) and inhibitory GABAergic and glycinergic interneurons.

The cerebellar nuclei provides cerebellar participation in a range of circuits by providing efferent connectivity via axon pathways to more caudal (from medial nuclei) and more rostral structure projections (from lateral nuclei) (Nichols and Bruce, 2006a). At the beginning, Altman and Bayer showed that a condensed nuclear transitory zone in the cerebellar primordium will be the primary location of the cerebellar nuclei neurons, which are originated from ventricular zone (Altman and Bayer, 1985c). Further studies revealed that only inhibitory neurons are originated from ventricular zone by mapping of Ptf1a receptor (Yamada et al., 2014) and excitatory cerebellar nuclei neurons have another origin, the rhombic lip, by mapping of Atoh1 receptor (Wang et al., 2005, Machold and Fishell, 2005).

Large glutamatergic projection cerebellar nuclei neurons

During early stages of cerebellar development, the excitatory cerebellar nuclei projection neurons (glutamatergic) originate from Atoh1-expressing neural progenitors, which arise from

the rhombic lip at around E9-12 (Marzban et al., 2015, Spencer et al., 2013). As explained before, the cerebellar nuclei neuron precursors migrate tangentially from the rhombic lip to the nuclear transitory zone in the rostral migratory stream (Fink et al., 2006c). In the development of glutamatergic cerebellar nuclei neurons, transcription factors Pax6, Tbr2, and Tbr1 are expressed sequentially within the neurons of the rhombic lip and nuclear transitory zone, a transient cell mass that is subsequently partitioned and organized to form the medial, interposed, and lateral cerebellar nuclei (Fink et al., 2006c). Studying *Tbr1* knockout mice revealed that there is similar number of cerebellar nuclei neurons, but the boundaries are not properly delineated (Fink et al., 2006c). Neuroblasts in the rhombic lip and rostral migratory stream are positive for Pax6/Reelin and move tangentially toward nuclear transitory zone to become Tbr2 positive cells (Fink et al., 2006c). The expression of Reelin is scattered in rostral migratory stream and studies on *reeler* mice showed no abnormalities in cerebellar nuclei progenitor migration itself. The abnormal and distorted cerebellar nuclei morphogenesis is due to abnormal Purkinje cell migrations (Fink et al., 2006c). In humans, the formation of the dentate nucleus (the largest and most lateral cerebellar nucleus) begins by stage 20 (52 days) when precursors (interneurons and projection neurons) migrate radially from the VZ and rostromedially from the rhombic lip (Marzban et al., 2015). In mice, projection neurons of cerebellar nuclei originate from the rhombic lip and unipolar neuroblasts migrate in the subpial stream toward the nuclear transitory zone under the guidance of both diffusible netrin1 (Ntn1, a member of a family of laminin-related secreted proteins) and slit (Slit Guidance Ligand, an extracellular matrix protein with role in bilateral symmetry) (Alcantara et al., 2000, Gilthorpe et al., 2002). The chemokine receptor 4 (CXCR4), a G-protein-coupled chemokine receptor, is broadly expressed in the CNS, and mediates migration in response to its ligand, SDF-1 (chemokine ligand 12 (CXCL12; also known as stromal derived

factor 1). During brain development, in the majority of brain regions (including cerebellar nuclei) the CXCR4/CXCL12 signaling pathway is the most important signaling pathway that regulates neuronal migration. This receptor was first seen in the immature neurons of the rhombic lip at E12.5 (Tissir et al., 2004). The expression of CXCR4 receptor continues in the rhombic lip-derived cerebellar nuclei precursors during the rostral subpial migratory stream pathway to the nuclear transitory zone. Their ligand, CXCL12, is simultaneously expressed in the meninges overlying this migratory pathway. Similar to tangential migration of granule cell precursor, this indicates the potential interaction of CXCR4- and CXCL12-expressing cells in the rostral migratory stream. This may facilitate the rostral migration of rhombic lip-derived neurons and directional change toward the nuclear transitory zone. This occurs through the detachment of neurons in this region from the pial surface to descend toward deeper positions in the nuclear transitory zone (Tissir et al., 2004).

Although there is some evidence for tangential migration of cerebellar nuclei neurons from the rhombic lip subpially to the midway of the cerebellar primordium, the mode of the migration and the substrate utilized during the change of the direction from the pial surface of the cerebellar primordium to the nuclear transitory zone are not known.

Granule cell

The granule cell precursors that originate in the rhombic lip (Atoh1 expressing progenitors) migrate tangentially through a subpial stream pathway known as the rostral migratory stream, and over the cortical surface to form the external germinal zone while expressing Pax6 (similar to rostral migratory stream from the ganglionic eminences to the olfactory bulb) (Komuro and Yacubova, 2003b, Stenman et al., 2003, Machold and Fishell, 2005). Simultaneously, granule cell precursors co-express Atoh1 and Barhl1 (BarH Like

Homeobox 1, Bar-class homeobox gene, *Mbh1*) in the rhombic lip and external germinal zone (Kawauchi and Saito, 2008). Although the existence of a substrate or scaffold in tangential migration has not been confirmed, it is possible that the pial meninges play a role as these processes are present underneath the pial surface (Komuro and Yacubova, 2003b). In addition, the external germinal zone is unique among proliferative germinal zones of the CNS as it is adjacent to the pial surface rather than the ventricular surface. The cells in this layer are highly proliferative, generating an enormous number of granule cell progeny, thereby greatly increasing the thickness of the external germinal zone. In mice at E12.5 to E17, granule cell precursors are born and migrate to establish the external germinal zone (postmitotic granule cells typically sojourn for 1–2 days within the lower layers of the external germinal zone) and give rise to the granule cells during the first two postnatal weeks (Wang and Zoghbi, 2001, Komuro et al., 2001). In human studies, it is shown that the external germinal zone is distinguished as a distinct layer between 10 weeks gestation to two month postnatally and will disappear by about year one and a half (Marzban et al., 2015). Granule cells initially follow a tangential migration and after proliferation in the external germinal zone, the cells migrate radially after Atoh1 expression stop. The granule cells situated in the inner layers of the external germinal zone start radial migration along Bergmann glial fibers to form the granular layer while expressing *NeuroD1*. The expression of *Unc5h3* and *Pax6* continues throughout the life span (Komuro and Yacubova, 2003a).Granule cells also change from a round cell to a more horizontal-oriented shape as they begin to extend axons tangential to the cortical surface. The CXCR4/ CXCL12 signaling pathway is involved in the migration of granule cell precursors in the rostral migratory stream from the rhombic lip. The alterations in this pathway result in the movement of granule cell precursors toward deeper positions away from the meninges, i.e., the inward radial migration, to

form the granular layer (Leto et al., 2016). In the granule cell migration pathway, semaphorin 6A (Sema6A) functions in the switch from tangential migration in the external germinal zone to radial migration along Bergmann glia (Leto et al., 2016).

These postmitotic granule cells migrate radially inward from the external germinal zone and pass by the developing Purkinje cells layer, to generate the granular layer. The cells migrate along the processes of the Bergmann glia, which is only present in the cortex of the cerebellum (Fig 1.8). Electron microscopic studies have detected Bergmann fibers in the external germinal zone by E15 in mice, and by 9 weeks gestation in humans (Choi and Lapham, 1980). The radial migration of the cerebellar granule neurons depends on actomyosin of the leading-process which coordinates organelle positioning and adhesion receptor dynamics (Ballif et al., 2004). During cerebellar development, doublecortin is strongly expressed by migratory granule cells (as occurs in Purkinje cells) to mediate coupling of the nucleus to the centrosome (Deutsch et al., 2010, Gleeson and Walsh, 2000). Shh which is expressed by Purkinje cells plays a key role in granule cell proliferation, and may also provide a stop signal for granule cell proliferation and the beginning of the terminal differentiation as these cells migrate toward the source of Shh in the Purkinje cell layer (Lewis et al., 2004). On the other hand, it is possible that the migration and maturation of granule cells is not associated with Shh pathway signaling, but rather the subsequent loss of precursor cells from the external germinal zone limits the period over which postmitotic granule cell precursors are generated (Lewis et al., 2004). In the study and review prepared by Yacubova and Komuro, they provided cellular and molecular mechanism involved in granule cell migration and they mentioned tangential migration of postmitotic granule cells in the middle and the bottom of the external germinal zone while their speed reduced from utter layer to inner layer and their process formed (Yacubova and Komuro, 2003, Yacubova and

Komuro, 2002). During their radial migration with days passing their speed increased but there is a stationary stage in Purkinje cell layer which follows by re-extension of granule cells somata and leading processes to reach granular layer (Yacubova and Komuro, 2003, Yacubova and Komuro, 2002).

In cerebellar granule cell migration, which is assisted by Bergmann glia, the majority of F-actin and myosin II is located at the front of the nucleus rather than the trailing end, suggesting these proteins may pull the soma forward (Solecki et al., 2009). The rate of cerebellar granule cells migration has been shown to be controlled positively through the frequency of the intracellular calcium fluctuation and negatively regulates the rate of extension of axonal growth cones (Bortone and Polleux, 2009). It was indicated besides calcium, NMDA (N-Methyl-D-aspartate) receptor also involved in granule cell migration. The guidance of granule cell migration is controlled by BDNF (brain-derived neurotrophic factor) and its high affinity receptor (TrkB, Tropomyosin receptor kinase B), neuregulin (NRG) and its receptor (ErbB4, Receptor tyrosine-protein kinase erbB-4) and EPHRIN (Borghesani et al., 2002, Rio et al., 1997, Lu et al., 2001). Somatostatin, a neuropeptide, plays a critical role in the migration of hematopoietic cells and seems to regulate cerebellar granule cells (Reisine and Bell, 1995).

Unipolar brush cell

Unipolar brush cells project directly to granule cells and amplify vestibular inputs to the cerebellum. In mice, the unipolar brush cells are generated from E13.5 to the early neonatal period (P0.5) (Marzban et al., 2015). These cells originate from the rhombic lip (as second wave between cerebellar nuclei neurons and granule cells genesis and are a third set of the rhombic lip derived neurons). Then they migrate through the developing white matter before settling in the granule cell layer (a unique type of glutamatergic interneuron in the granule cell layer) of the

cerebellar cortex (Englund et al., 2006a). Englund et al. reported that Tbr2 positive unipolar brush cells migrated from rhombic lip explants directly into the developing white matter of adjacent cerebellar slices (Englund et al., 2006a). Unipolar brush cells project directly to granule cells and amplify vestibular inputs to the cerebellum (Hevner et al., 2006). The translocation of the unipolar brush cells from white matter to granule cells occurs between P3 to P10 and these cells sojourn in white matter for a period of time (Englund et al., 2006a). Loss of the *Nf1* (neurofibromatosis type 1) gene leads to imbalance in generating the appropriate numbers of glia and controlling granule cell/unipolar brush cell fate-specification/differentiation, and granule cell/unipolar brush cell migration (Kim et al., 2014). Unipolar brush cells express Tbr2 throughout life, from genesis in the rhombic lip to adulthood, and used widely as a unipolar brush cell marker (Mihalas and Hevner, 2017, Yeung and Goldowitz, 2017, Englund et al., 2006a). Tbr2 may be involved in the migration and differentiation of unipolar brush cells which must be examined in Tbr2 knockout mice. However, neither unipolar brush cells nor their progenitors express Tbr1, which express in cerebellar nuclei neurons (Mihalas and Hevner, 2017). Interestingly, Yeung and Goldowitz are shown that Tbr2 positive unipolar brush cells are significantly reduced (~85%) in the Wls-cKO (Wntless-conditional knockout, WLs regulates Wnt1secretion at the isthmus organizer which is required to induce midbrain–hindbrain structures) cerebellum. They have shown that this reduction in Tbr2 positive cells in the cKO is uniform and does not exhibit a medial-to-lateral effect, and Wls-expressing cells in the rhombic lip are involved in the cerebellar development coordination (Yeung and Goldowitz, 2017). Furthermore, Wnt1 glycoprotein expression in the upper rhombic lip and unipolar brush cells is related to molecular identity and cell migration in cerebellar development (Hagan and Zervas, 2012). The role of Lmx1a in unipolar brush cells need to be addressed in *Lmx1a* null mice

(*dreher* mutant) while it is shown that there is no significant changes in unipolar brush cell genesis (Chizhikov et al., 2010b). Studies have also shown a role for doublecortin in the neurogenesis and migration of unipolar brush cells (Paolone et al., 2014, Manohar et al., 2012).

1.6.2 Ventricular zone derived cells

Purkinje cells

Purkinje cells, which are key neurons in cerebellar cortex networking, complete their final mitotic division at E10–13 in mice (Hashimoto and Mikoshiba, 2003, Minaki et al., 2008, Hashimoto and Mikoshiba, 2004). Once in the postmitotic stage, they start a short distance radial migration along the radial glial fibers (scaffold/substrate) from their site of origin in the ventricular zone (Hatten, 1999, Rakic and Sidman, 1970, Yuasa et al., 1996). Purkinje cells exhibit an elongated morphology consistent with axonogenesis at E12.5, which is directed toward the mantle zone where they accumulate as irregular multilayer cells called the cerebellar plate, or Purkinje cell plate at E14.5 (Miyata et al., 2010). During the early stage of Purkinje cell migration, Purkinje cells initially have a leading apical neurite and trailing process, and the cell's position changes along with cerebellar primordium expansion and morphological development (Sotelo and Dusart, 2009, Hatten and Heintz, 1995). Conventionally it is thought that the newborn Purkinje cells from the rostral ventricular zone migrate radially, along the processes of radial glia, while those that originate from caudal ventricular zone first migrate tangentially and upon arrival to destination, initiate a second phase of radial migration to form the Purkinje cell plate (Miyata et al., 2010, Sotelo, 2017). It is indicated that some Purkinje cells' migration is clearly crossing the radial fibers, not along them, and therefore they are non-radial migrating Purkinje cells. The translocation of Purkinje cells along radial glia has never been observed in live preparations and is based on still images (Schilling, 2018, Miyata et al., 2010). There is no

convincing evidence for the latter approaches; however, it can be speculated that despite the tangentially oriented Purkinje cells in position some of these cells use radial glial fibers for migration. The fast proliferating ventricular zone neuroepithelum leads to prominent bulging toward the 4th ventricle and caudal appearance of the cerebellar primordium seems oriented horizontally, however radial glial fibers are connected from neuroepitheiulm to the subpial surface and provide substrates for the Purkinje cells originated from the caudal ventricular zone.

After ~E14.5 the Purkinje cell plate, a multi-cell-thick immature Purkinje cell layer, is expanded in orchestra with the cerebellum development and aggregated in several Purkinje cell clusters that become well established at around E17.5 (Fujita and Sugihara, 2012). It appears that there is no migratory activity during this stage, but rather a small displacement of Purkinje cells groups following expansion of the developing cerebellum.

The second wave of Purkinje cell migration is initiated after formation of the Purkinje cell cluster. Purkinje cells disperse and arrange in a single regularly spaced monolayer during cerebellar expansion and simultaneously grow their axon and dendrites (Butts et al., 2014). During Purkinje cells differentiation, they collapse their apical neurite (at P0) and form numerous short neurites at ~P6 which develop ramified dendrites at ~P8 (Armengol and Sotelo, 1991). In human studies, it is shown that Purkinje cell progenitors start their migration from the ventricular zone at stages18 and 19 of the development (~ 44–48 days) (Müller and O'Rahilly, 1990). They form a monolayer at 16–28 weeks of the gestation, and start enlargement with the development of more dendritic branches, which is associated with an increase in synapse formation (Müller and O'Rahilly, 1990).

It is not clear how Purkinje cells disperse from the cluster stage to the monolayer position. One of the most studied molecules that is involved in the control of Purkinje cell

migration is Reelin (Larouche and Hawkes, 2006). During mouse cerebellar development, the expression of Reelin (*Reln* mRNA and glycoprotein) is first detectedin the dorsal cerebellar surface,the rhombic lip-derived cells zone and the nuclear transitory zone at E13 (Fink et al., 2006c). By the first week of postnatal life, Reelin expression disappears from the deep cerebellar regions, but is maintained in granule cell precursors. Also,the expression of Reelin may continue in some granule cells of adult mice (Fink et al., 2006c). The dispersal of Purkinje cells from clusteric stage depends on Reelin expression and its downstream molecules ApoER2 (apolipoprotein E receptor 2) and VLDLR (very-low-density lipoprotein receptor). Reelin binds with similar affinity to ApoER2 and VLDLR. Purkinje cells express Disabled-1 (Dab1), which is a key molecule in the Reelin pathway, and settle underneath the Reelin-expressing cells of the external germinal zone (Miyata et al., 2010, Fatemi, 2005).

Mutations in the *Reelin* gene (*reeler* mouse) (D'arcangelo et al., 1995), *dab1* gene (scrambler and yotari mouse) (Howell et al., 1997, Sheldon et al., 1997), and targeted deletion of the genes for both *ApoER2* and *VLDLR* (Trommsdorff et al., 1999) all result in a similar phenotype of ectopic Purkinje cells due to a lack of dispersal from the cluster. These findings have placed Reelin, ApoER2, VLDLR, and Dab1 into a common signaling pathway. It has been shown that Reelin protein resembles extracellular matrix proteins that are involved in cell adhesion (D'arcangelo et al., 1995) and regulates cadherin function via Dab1. Cadherins are a group of trans-membrane proteins that mediate cell–cell adhesion during tissue morphogenesis (Maître and Heisenberg, 2013). Cadherin-6B (also known as cadherin-7) is overexpressed in Purkinje cell progenitors and is involved in the guidance of migrating neurons along neurites that express the same cadherin during their radial migration. These findings indicate that cadherin adhesive mechanisms are involved in neuronal guidance (Takei et al., 2000). In addition, it has

been shown that Dab1 signaling controls the adhesive ability of neurons to radial glia. The newborn neurons in the cortex of *scrambler* mice remain attached to the process of their parental radial glia during the entire course of radial migration. This abnormal neuronal-glial adhesion is highly linked to the positional abnormality of neurons in scrambler mice. Additionally, the phosphorylation of tyrosine residues of Dab1 regulates α3 integrin levels in migrating neurons and their timely detachment from the radial glial fibers (Sanada et al., 2004). Furthermore, direct interaction of phosphorylated Dab1 with other intracellular proteins such as members of Crk (adapter molecule crk also known as proto-oncogene c-Crk or p38) family might connect the Reelin pathway to integrin-mediated adhesion and migration of neurons (Ballif et al., 2004, Mayer et al., 2006). It has been shown that abnormal migration of neurons in *scrambler* mice is associated with an impaired detachment of neurons from clonally related radial glial cells. This abnormal neuronal-glial adhesion depends on α3 integrin signaling that is regulated by Dab1 (Sanada et al., 2004). Reelin clearly plays an important role in Purkinje cells migration and monolayer formation of the Purkinje cell layer during postnatal cerebellar development, but still around 5% of Purkinje cells migrate appropriately independent of the Reelin pathway (Trommsdorff et al., 1999). Yasua et al also introduced that lateral Purkinje cells start to migrate very early (at around E10.5) while at this time-point cerebellar Reelin expression has not yet started and they conclude that delamination of postmitotic Purkinje cells from the ventricular neuroepithelium and their initial migration is independent of Reelin signaling and radial glia (Yuasa et al., 1993). However, a major challenge still remains: how do Purkinje cells from the clusteric stage disperse to their position in a monolayer? Do Purkinje cells use any substrates such as Bergmann glia cells to migrate from the clusteric stage to their final destination?

Bergmann glia are defined as Purkinje cell-associated astrocytes, and are a specific type of astrocyte in the cerebellar cortex. Bergmann glial cells originate from radial glia within the ventricular zone. The radial glia transform to Bergmann glial cells during E14.5 - E18.5 in mice under control of the Ptpn11, which maintains the basal processes of radial glia and relocates somata from the ventricular zone to the nascent Purkinje clusters (Yuasa, 1996, Li et al., 2014). It is well documented that Bergmann fibers are associated with granule cells in migration during cerebellar postnatal development, and this is the origin of the concept of glia-guided neuronal migration (Hatten, 1990). Recently, the role of Bergmann glia in the generation of cerebellar folia was investigated (Leung and Li, 2017). It was suggested that Bergmann glial cells are essential in cerebellar corticogenesis, especially through monolayer formation of Purkinje cells and dendritogenesis, granule cells migrations, and circuit formation (Rakic, 1971, Altman and Bayer, 1997, Cajal, 1911, De Zeeuw and Hoogland, 2015). Yamada et al. showed that not only are Bergmann glial cells associated with Purkinje cells in the adult cerebellum but they are also associated with Purkinje cells during their migration (their somata leave ventricular zone slightly just after Purkinje cells), dendritogenesis, synaptogenesis and maturation (Yamada et al., 2000, Yamada and Watanabe, 2002). At E13, the somata of cerebellar radial glial cells - the precursor of Bergmann glia – are aligned along the ventricular surface of the fourth ventricle, and they extend processes up to the pial (meningeal) surface of the cerebellar plate. By the translocation of radial glial somata from ventricular zone, the delamination and migration of Purkinje cells is tightly followed with the detachment of the trailing process of the radial glia from ventricular zone at E18 (Yuasa et al., 1996). There is controversy about role of Bergmann glia in Purkinje cell monolayer formation through Notch-RBP-J signaling and notch ligand, Delta-like 1(Komine et al., 2007, Hiraoka et al., 2013) while the ablation of these genes from Bergmann glia was not

affected Purkinje cell monolayer formation, the ablation of Dner (delta/notch-like EGF receptor containing) from Purkinje cells result in Bergman glia disruption (Greene et al., 2016, Tohgo et al., 2006, Eiraku et al., 2005). Therefore, it is possible that Purkinje cell utilize a new mode of migration in which Purkinje cells migrate passively with the assistance of a pulling force from the Bergmann glia by regulating cells adhesion molecules to form a Purkinje cell monolayer. This should be examined in future studies.

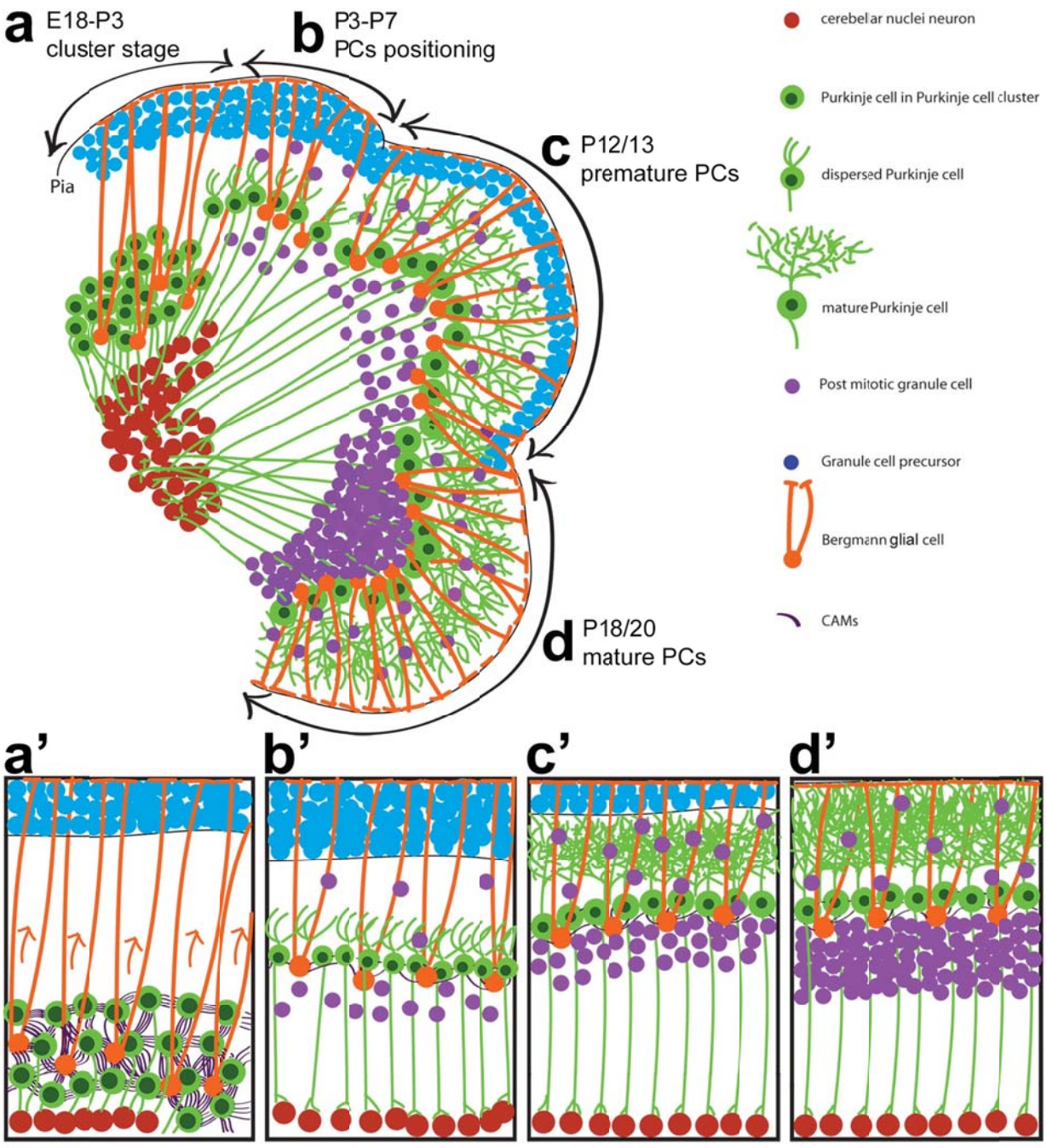

Figure 1. 8 Neuronal migration during postnatal cerebellar development

A schematic illustration of cerebellar development at sagittal section of postnatal mice. a, a') It shows that Purkinje cells cluster disperse in the monolayer and start maturation while granular layer form from external germinal zone (E18-P20). a, a') Cluster stage is between E18 to P3, Purkinje cells in clustering situation show with high CAMs (cell adhesion molecules)

connections and shows long Bergmann glia beside Purkinje cell cluster extend fibers to pia matter. b, b') Purkinje cells in dispersal situation and positioning process (P3-P7) with less CAMs connections and shows shortened Bergmann glia beside monolayer Purkinje cells. c, c') It shows how premature Purkinje cells (P12/13) arborized (maturation process) and become mature Purkinje cells while granule cell precursors migrate inwardly (away from pia) and become mature granule cells. (d, d'); Purkinje cells become matured (P18/20) while and granule cell precursors start continue inward migration and complete granular layer(away from pia) and become mature granule cells. P: postnatal day, E: embryonic day, CAMs: cell adhesion molecules.

Intermediate GABAergic projection cerebellar nuclei neurons

The developmental origin of GABAergic projection (nucleo-olivery) neurons is vague, although it is assumed they are originated from ventricular zone and they are first wave of ventricular zone originated GABAergic neurons and express Ptf1a (followed by production of Purkinje cells and interneurons) (Sudarov et al., 2011). Postmitotic GABAergic projection cerebellar nuclei neurons are expressing Neurog1 (Zordan et al., 2008). Using visualization of IRX3 (Iroquois homeobox gene family 3 (Zordan et al., 2008)) positive cells, Morale *et al.* revealed a columnar arrangement of the progenitors of the cerebellar nuclei migrating from the ventricular zone to the surface along the radial glial fibers. At E12.5, increasing numbers of IRX3 neurons opposed to radial glial fibers and they have assumed that the radial glial fiber system appears to provide the migratory pathway for the initial migration of cerebellar nuclear progenitors from the VZ to the surface of the anlage (Morales and Hatten, 2006).

Small GABAergic cerebellar nuclei neurons

Small GABAergic cerebellar nuclei neurons such as Purkinje cells express Neurog 2, so the expression of Neurog1 and Neurog2 defines the Ptf1a positive ventricular zone originated population from Pax2-positive precursors (Florio et al., 2012). Similar to other GABAergic interneuron precursors, they are generated from ventricular zone progenitors at E13.5-16.5 (Pierce, 1975) and in *Ptf1a* mutant (cerebelless) mice GABAergic positive neurons are rarely seen (Hoshino et al., 2005). GABAergic cerebellar nuclei neurons express Ptf1a/Pax2/ Gsk1 and migrate through white matter to settle down in cerebellar nuclei (Seto et al., 2014b, Leto et al., 2006, Yamada et al., 2014). Mutation of Purkinje cell progenitor transcription factors Olig2 and Gsx1 disrupts the production of Pax2 lineages suggesting that the latter is derived from the former during development (Seto et al., 2014b). There are several issues to be investigated about machinery controlling small GABAergic cerebellar nuclei neurons; is Irx3 involved? Is there any room for glial pathways? In addition, the origin and development of the various types of glycinergic neurons in the cerebellar nuclei have yet to be characterized.

GABAergic interneurons (stellate/basket, and Golgi cells)

Precursors of stellate and basket cells are generated from the ventricular zone prenatally (when they express the paired homeobox gene, Pax2) and then migrate from the cerebellar plate to the developing white matter and postnatally (in mice) through the folial white matter while continuing to undergo cell division (Wefers et al., 2018). Thereafter, they migrate radially towards the molecular layer to accumulate at the inner border of the external granular layer and then migrate tangentially before settling at their final position, and are distributed within the molecular layer. A new study published recently by Wefers et al. documented the movement of the cerebellar interneurons, basket and stellate cells, are highly directed and rerouted to the

molecular layer during their transit through the nascent cerebellar cortex (Wefers et al., 2018). In this study, it has been shown that the speed and directional persistence of basket and stellate cells are larger in the nascent granule cell layer than in the molecular layer (Wefers et al., 2018).

Golgi cell precursors are granule cell layer inhibitory interneurons, and are born within the ventricular zone prenatally (E12-15). From the cerebellar plate, these cells migrate to developing white matter while continuing proliferation during migration until around P4 (Zhang and Goldman, 1996, Weisheit et al., 2006). During perinatal development, Golgi cells precursors continue the migration through the developing folial white mater and terminate migration postnatally by positioning in the developing granular layer (Maricich and Herrup, 1999). In addition, a subset of Golgi cells are derived from the external germinal zone (Chung et al., 2011). This subset of cells migrates within the white matter and postnatally becomes postmitotic and then migrates to position within the granular layer (Wefers et al., 2018, Yamanaka et al., 2004).

Although the mode and direction of GABAergic interneurons of the cerebellar cortex migration is a complex process and is not entirely clear from origin to final position, but based on the evidences, it could be with a lot of changes from radial to tangential migration within the cerebellar cortex.

CHAPTER TWO: THESIS OVERVIEW

2.1 Rational for the first part of the project:

Formation of the cerebellar nuclei neurons:

Cerebellar nuclei are made up of at least three neuronal types: large excitatory glutamatergic projection neurons, inhibitory GABAergic projection (to inferior olive) and interneurons, and glycinergic neurons (probably inhibitory projection and interneurons) (Voogd, 1992, Mugnaini, 1985, Englund et al., 2006b, Fink et al., 2006b, Bagnall et al., 2009, Marzban et al., 2014). Glutamatergic neurons are born around E9-E12 in mice (Miale and Sidman, 1961), while GABAergic interneuron precursors that express the transcription factor Pax2 are produced around E12-E15 from the ventricular zone (Maricich and Herrup, 1999). Although it is suggested that glycinergic projection neurons (located in medial nuclei) and small glycinergic interneurons that are dispersed throughout the cerebellar nuclei arise probably from the ventricular zone (Helms et al., 2001), the birth-dating of this cerebellar nuclei subpopulation is not clear.

The germinal zone for the glutamatergic and glycinergic subset of cerebellar nuclei neurons is not well defined. Previously, it was believed that glutamatergic and GABAergic cerebellar nuclei neurons originate from a common germinal zone, i.e. the neuroepithelium of the fourth ventricle (the ventricular zone) (Altman and Bayer, 1985d, Altman and Bayer, 1985b, Altman and Bayer, 1985a). However, recent birth-dating and genetic fate mapping studies suggested that the rhombic lip may produce the majority of cerebellar nuclei projection neurons (Fink et al., 2006b, Ben-Arie et al., 1997b, Machold and Fishell, 2005). The transcription factor expression patterns suggest that cerebellar nuclei neurons migrate from the rhombic lip to the nuclear transitory zone (NTZ) through a subpial stream pathway while sequentially expressing the genes Pax6, Tbr2 and Tbr1 (Fink et al., 2006b, Marzban et al., 2014).

In the cerebellum, Tbr1 in the glutamatergic component of cerebellar nuclei neurons appears to play an important role during early cerebellar development. Tbr1 regulates Reelin, a protein that is involved in neuronal migration and positioning (Jensen et al., 2004). Studies using mutant mice for the genes *Tbr1* and *Reln* (*reeler*) have helped to determine whether Tbr1 or Reelin are necessary for cerebellar nuclei migration and development. Interestingly, both mutant strains revealed defects of cerebellar nuclei morphogenesis, although neither gene appears to be required for cell migration from the rhombic lip to the NTZ domain (Fink et al., 2006b).

It has been shown that Lmx1a is an important regulator of cerebellar nuclei neurogenesis originating from rhombic lip and is also expressed in the NTZ (Chizhikov et al., 2010a, Chizhikov et al., 2006b). Despite the role of Lmx1a in the rhombic lip, it was still expressed in Math1 null embryos, which may suggest that some $Lmx1a^{+}$ neurons originate from the Math1-independent population (Jensen et al., 2004, Yeung et al., 2014) or that they do not arising from the rhombic lip (Chizhikov et al., 2010a). It has also been shown that $Tbr1^{+}$ neurons are also present in cerebellar primordium with an ablated rhombic lip (Fink et al., 2006b). This indicates that probably not all cerebellar nuclei neurons arise from the rhombic lip. Reinforcing this idea is that in some cerebellar anomalies affect merely a subdivision of medial cerebellar nuclei. This suggests that Tbr1 and/or Lmx1a may control only a subset of the medial cerebellar nuclei. To further explore cerebellar nuclei development, we used anti-α-Synuclein (SNCA; a lipid-associated protein) antibody, which is expressed in the somata of Purkinje cells, cerebellar nuclei neurons and axon terminals, and also interacts with proteins such as tau, tubulin, and actin (Zhong et al., 2010b, Vekrellis et al., 2004, Esposito et al., 2007). Our preliminary data, based on SNCA expression, revealed a subset of $SNCA^{+}/Lmx1a^{-}$ CN neurons. $SNCA^{+}$ neurons run from the dorsal mesencephalon and cross the isthmus to the NTZ as early as E9. Using double

immunocytochemistry, we showed that an Lmx1a$^+$ population in the NTZ flanks a subset of neurons that express SNCA. The majority of neurons are not SNCA$^+$/Lmx1a$^+$ but some of the neurons are co-labeled. This suggests that in the early stages of cerebellar nuclei neurogenesis, the pattern of gene expression in SNCA$^+$ neurons is changing, possibly with SNCA being down regulated and Lmx1a$^+$ is being up regulated. In addition, using SNCA and NAA 3A10 (a neurofilament associated antigen (Marzban et al., 2008b)) at E9 revealed a number of SNCA$^+$ and NAA$^+$ large neurons present in the NTZ before any neurons arrive from the rhombic lip. This suggests that a subset of cerebellar nuclei neurons in the NTZ domain arise from an as-yet-unidentified germinal zone. Taken together, the origin, position, fate, and role of these large neurons within the cerebellum is currently unknown. My data strongly support the idea that there may be a third germinal zone, which forms a subpopulation of cerebellar nuclei neurons that likely predates all neurogenesis in the cerebellar primordium that appears to be crucial for normal cerebellar development (Marzban et al., 2014). Interestingly, this subpopulation seems to arise from the mesencephalon (extrinsic germinal zone) but at present, it is not clear if this new third germinal zone is derived from the neuroepithelium or from neural crest cells, and this will be addressed in first part of my study.

2.2 Hypothesis for the first part of the project:

I hypothesize that an early subset of mouse cerebellar nuclei neurons originates from the mesencephalon and mesencephalic neural crest and are essential in cerebellar development.

2.3 Specific aims for the first part of the project:

Specific Aim #1- To investigate the source of the early subset of CN neurons in the developing mouse cerebellum

Specific Aim #2- To examine the fate of the early subset of CN neurons during development

Specific Aim #3- To determine the role of the SNCA in early subset of CN neurons during cerebellar development

2.4 Rationale for the second part of the project:

As I will explain in chapter three, we have found a subset of neurons appears to originate from the mesencephalon (a new germinal zone) during cerebellar development. Our findings were shown that this subset of neurons derived from mesencephalon is positive for "protein 75 neurotrophin receptor" (p75NTR) in nuclear transitory zone and also in the neuronal and nerve fibers at the future putative VI and VII lobules of the cerebellar primordium when labeled with this antibody as early as E9.5 in mice. These observations triggered question whether the distribution and pattern of p75ntr expression can be traced during perinatal cerebellar development. In addition, as Lysosomal Acid Phosphatase 2" (*Acp2*) gene is expressed in mesencephalic derive cerebellar nuclei neurons during early development, we used Acp2 mutant mice cerebellum in this study as well. Lysosomal acid phosphatase 2 (Acp2) is a key enzyme in cerebellar development, and this mutant mouse shows severe cerebellar defects including ataxia, reduced size with neuronal migration abnormalities and abnormal lobulation as well as Purkinje cell degeneration.

2.5 Hypothesis for the second part of the project:

I hypothesized that there is a difference in p75NTR cerebellar cortex expression during the early postnatal days in adult cerebellum in comparison to *Acp2* mutant mice

2.6 Specific aims for the second part of the project:

Specific Aim #1- To determine pattern of the p75NTR expression in postnatal mouse cerebellum

Specific Aim #2: - To analyze the alteration of the pattern of the p75NTR expression in Acp2 mutant mouse cerebellum postnatally.

CHAPTER THREE: EARLY SUBSET OF CEREBELLAR NUCLEI NEURONS DERIVED FROM MESENCEPHALON IN MICE

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3.1 Introduction

The cerebellum is involved in a variety of brain functions including motor and non-motor functions (Glickstein et al., 2009, Thach, 2007). Cerebellar nuclei (CN) are the main structures responsible for cerebellar output channels (Voogd and Glickstein, 1998). CN plays a pivotal role in integrating signals, information processing, and cognition (Strick et al., 2009, Ivry, 1996). During the embryonic stage, CN and Purkinje cells (Pcs) are the only neuronal populations that exist in early cerebellar development (Voogd and Glickstein, 1998, Voogd and Ruigrok, 1997, Marzban et al., 2015). In the mouse, the cerebellar primordium emerges at approximately embryonic day (E) E7–E8 as a neuroepithelial swelling on the rostral lip of the fourth ventricle, which is part of the alar plate of the metencephalon (rhombomere-1) (Goldowitz and Hamre, 1998, Sotelo, 2004, Wang and Zoghbi, 2001, Marzban et al., 2015). The cerebellar primordium contains two distinct germinal zones which are responsible for cerebellar neurogenesis and gliogenesis including the ventrally-located ventricular zone (VZ) and dorsally-located rhombic lip (Fink et al., 2006a).

For CN development, early studies demonstrated that both glutamatergic and GABAergic CN neurons originate from the cerebellar VZ (Voogd and Glickstein, 1998). Further studies highlighted the involvement of the rhombic lip as an origin for glutamatergic CN neurons (between E9-E12) (Fink et al., 2006a, Wang and Zoghbi, 2001, Green and Wingate, 2014). To investigate the origin of CN during the cerebellar embryonic stages, our data suggest that the mesencephalon may be a novel origin of newly characterized neurons that contribute to CN formation and development. In this context, the earliest neuronal outgrowth during central nervous system (CNS) development occurs in E9 in the mesencephalic nucleus of the trigeminal nerve (Stainier and Gilbert, 1989, Rahimi-Balaei et al., 2015a). Neurons in this region are

considered the large sensory neurons and are derived from the neural crest cell (NCC) population (Jacobson, 2013, Narayanan and Narayanan, 1978b). At the embryonic stage, NCCs arise from the transient neural crest that initially generates neural crest stem cells, in which migrating cells express the early neural crest marker, p75 neurotrophin receptor or p75ntr (Lee et al., 2002, Schwarz et al., 2008). Neuronal proliferation/differentiation is regulated by p75ntrs that are mainly expressed during early development (Jiang et al., 2008, Bernabeu and Longo, 2010a, Dechant and Barde, 2002b). In addition, previous studies suggest that the orthodenticle homeobox 2 (*Otx2*) plays an essential role in the development of the mesencephalic neural crest-derived neurons and also the cells of the rhombic lip choroid plexus (Kurokawa et al., 2004, Kimura et al., 1997, Hagan and Zervas, 2012). It is also indicated that the mesencephalic NCCs express *Otx2* during their migratory phase (Kimura et al., 1997).

The LIM homeobox transcription factor 1, alpha (Lmx1a), however, is an important regulator of CN neurogenesis which originates from the rhombic lip. It is also expressed in the nuclear transitory zone (NTZ) and is considered to be a marker for majority of rhombic lip-derived CN neurons beside subset of the Lmx1a positive cells that do not originate from the rhombic lip migratory stream (Chizhikov et al., 2006a, Chizhikov et al., 2010b). In this study, we hypothesized that there is a subset of cells with NCC characteristics that migrate from the mesencephalon and play an important part in CN development. To do this, we studied Otx2, p75ntr, and Lmx1a in the developing cerebellum (E9-E18). Our findings suggest that the mesencephalon may be a third germinal zone that is crucial for the vermis and anterior cerebellar development. Additionally, we studied α-synuclein (SNCA; a lipid-associated protein), which is expressed in the somata of CN neurons and axon terminals in the cerebellum, and interacts with proteins such as tau, tubulin, and actin (Zhong et al., 2010b, Vekrellis et al., 2004, Esposito et al.,

2007). We evaluated SCNA because it is considered to be involved in the development of neural stem cells and synaptogenesis in developing neurons (Zhong et al., 2010b, Rogers and Schor, 2010a).

3.2 Material and Methods

Animal maintenance

All animal procedures were performed in accordance with institutional regulations and the *Guide to the Care and Use of Experimental Animals* from the Canadian Council for Animal Care. In this study, we used embryos from 47 CD1 timed-pregnant mice at E9 to E18 (total embryo number was 409), three CD1 mice at postnatal day (P) P4. In this study, our approach for sample size was to continue to include further samples until saturation is reached. The number needed to reach saturation has been investigated empirically (in most of the experiments n was equal to 9).

All timed-pregnant CD1 mice were obtained from the Central Animal Care Service, University of Manitoba. Animals were kept at room temperature and relative humidity (18–20°C, 50–60%) on a light and dark cycle (12:12 h) with free access to food and water. The embryo age was determined from the first appearance of a vaginal plug, considered E 0.5. CD1 timed-pregnant mice at E (9, 10, 11, 12, 13, 14, 15, 16 and 18) + 0.5 (N=47, see table 3.1) were anesthetized [40% isoflurane, USP (Baxter Co. Mississauga, Ontario, Canada)] and embryos were removed and fixed in 4% paraformaldehyde (PFA) for immunohistochemistry (IHC) or prepared for Western Blotting. Three CD1 mice at P4 were transcardially perfused with PBS for IHC, as described previously (Bailey et al., 2014a, Bailey et al., 2013a). In this study like "Sample size determination in qualitative studies" I took a different approach. It is generally a

subjective judgment, taken as the research proceeds. One approach is to continue to include further participants or material until saturation is reached. The number needed to reach saturation has been investigated empirically.

CD1 T.P	#	Total #
E9	5 WB+IHC/IF, 2 EC (DiI), 2 EC (Q-tracker), 4 EC (fast DiI), 1 EC (Hoechst 33342)	14
E10	3 WB+IHC/IF , 3 DCC	6
E11	3 WB+IHC/IF	3
E12	3 WB+3IHC/IF+3RS	9
E13	3 WB+IHC/IF	3
E14	3 IHC/IF	3
E15	3 WB+IHC/IF	3
E16	3 IHC/IF	3
E18	3 WB+IHC/IF	3
		47

Table 3. 1 Sample size for the number of CD1 time pregnant used for different experiments

T.P. = Time Pregnant, E= Embryonic Day, WB= Western Blotting, IHC= Immunohistochemistry, IF= Immunofluorescence, EC= Embryonic Culture

DCC= Dissociated Cerebellar Culture, RS= RNAscope, Ab= Antibody

CD1 Embryo	#	*Total #*
E9	35 WB+ 12 IHC/IF+ 16 EC (DiI), 18 EC (Q-tracker), 40 EC (fast DiI), 8 EC (Hoechst 33342)	129
E10	21 WB+ 9 IHC/IF , 29 DCC	59
E11	20 WB+9 IHC/IF	29
E12	31 WB+ 28 IHC/IF (target Ab and Ab optimization+ 35 RS	94
E13	20 WB+ 9 IHC/IF	29
E14	9 IHC/IF of total 36	9 used for IHC (27 are stored in 4% PFA)
E15	18 WB+ 9 IHC/IF	27
E16	9 IHC/IF of total 30	9 used for IHC (21 are stored in 4% PFA)
E18	15 WB+ 9 IHC/IF	24
		409 embryos

Table 3. 2 Sample size for the number of CD1 embryos used for different experiments

T.P. = Time Pregnant, E= Embryonic Day, WB= Western Blotting, IHC= Immunohistochemistry, IF= Immunofluorescence, EC= Embryonic Culture, DCC= Dissociated Cerebellar Culture, RS= RNAscope, Ab= Antibody

Sections immunohistochemistry (IHC)

Cryostat sections (20 μm) of PFA fixed samples were attached to slides and prepared for IHC process as explained in our previous studies (Bailey et al., 2014a, Bailey et al., 2013a). Antibody dilutions were used as follows: α-synuclein (sc-69977, Santa Cruz) 1:500, p75NTR (8238, Cell Signaling) 1:1000, Lmx1a (AB10533, EMD Millipore Corporation) 1:500, Otx2 (ab114138, abcam) 1:1000, NAA (3A10, Developmental Studies Hybridoma Bank) 1:500, βTubulin (T8328, Sigma-Aldrich) 1:2000, TrkA (ab76291, abcam) 1:50, and Cleaved Caspase 3 (9664, Cell Signaling) 1:200. Fluorescent detection was performed using antibodies as follows: Streptavidin, Alexa Fluor® 488 conjugate, Alexa Fluor® 568 Goat Anti-Rabbit IgG (H+L), Alexa Fluor 488 Chicken Anti-Mouse IgG (H+L), Alexa Fluor 488 Chicken Anti-Rabbit IgG (H / L), and Alexa Fluor 568 Goat Anti-Mouse IgG (H+L) (S-11223, A-11036, A21200, A21441, A11004 Life Technologies) 1:1000. Detection of peroxidase IHC was also performed as described previously (Rahimi Balaei et al., 2016b, Bailey et al., 2014a, Bailey et al., 2013a) using HRP conjugated goat anti-rabbit IgG and goat anti-mouse IgG (H+L) antibodies (EMD Millipore Corporation, 12-348 and AP308P, respectively) 1:500, and developed with DAB (3,3'-diaminobenzidine) solution (Sigma, St. Louis MO, USA).

Otx2 positive cell counting

To study the number of Otx2 positive cells and count them, slides with the sections of E12, 13, 14 and 15 were labeled for Otx2. Under microscope the total Otx2 positive cells in cerebellar primordium were counted in each section of the slide. The number of positive cells in each section were added to each other in one slide and prepared for statistical analysis.

Whole mount IHC

Whole mount IHC was performed on embryos according to Sillitoe and Hawkes (Sillitoe and Hawkes, 2002) with a few modifications. Briefly, PBS containing 0.2% skim milk (Nestlé Foods Inc., North York ON, Canada) plus 0.1% Triton-X 100 (Sigma, St. Louis MO, USA) and 5% dimethyl sulfoxide (DMSO) at 4ºC overnight was used as the blocking solution (PBSMT). After primary and secondary incubation, the immunoreactivity was revealed by DAB, and then the tissue was washed in PBS and stored in PFA for analysis.

Primary cerebellar cell culture

Primary cerebellar cultures were prepared from embryonic (E) day 10 CD1 mice, and cells were maintained for varying days *in vitro* (DIV 1, 2, 3, 5, and 8), according to published methods (Bailey et al., 2013a). Briefly, the entire cerebellum was removed from each embryo and immediately placed into ice cold Ca^{2+}/Mg^{2+}-free Hank's balance salt solution (HBSS) containing gentamicin (10 μg/ml) and glucose (6 mM). The cerebella were incubated at 34°C for 12 min in HBSS containing 0.1% trypsin. After washing, the cerebella were gently triturated in HBSS containing DNase I (5 U/ml) and 12 mM $MgSO_4$ until the cell mass was no longer visible. The cells were collected by centrifugation (1,200 rpm, 4°C for 5 min) and re-suspended in seeding medium (1:1 Dulbecco's modified Eagle's medium and F12) supplemented with putrescine (100 μM), sodium selenite (30 nM), L-glutamine (1.4 mM), gentamicin (5 μg/ml), and 10% heat-inactivated fetal bovine serum. The cell suspensions were seeded on poly-L-ornithine coated glass coverslips (12 mm) at a density of 5×10^6 cells/ml, with each coverslip placed into the well of a 24-well plate. After 6–8 h incubation in a CO_2 incubator (100% humidity, 37°C, 5% CO_2), 500 μl of culture medium supplemented with transferrin (200 μg/ml), insulin (20 μg/ml),

progesterone (40 nM), and triiodothyronine (0.5 ng/ml) was added to each culture well. After 7 days, half of the medium in each dish was replaced with fresh medium that was additionally supplemented with cytosine arabinoside (4 μM) and bovine serum albumin (100 μg/ml) (Bailey et al., 2013a, Marzban and Hawkes, 2007, Marzban et al., 2003b).

Embryonic cultures and DiI labeling of cells within the mesencephalon

Embryonic cultures were prepared from E9 and E10 CD1 timed-pregnant mice, and embryos were maintained for various DIV (4, and 6). Each embryo was removed from the amniotic sac and immediately placed into ice cold Ca^{2+}/Mg^{2+}-free HBSS containing Gentamicin (10 μg/ml) and glucose (6 mM). Embryos were placed into 24-well plates in culture medium plus 10% fetal bovine serum and incubated in a CO_2 incubator (100% humidity, 37°C, 5% CO_2) (Marzban and Hawkes, 2007). Embryos were monitored every 6 h to evaluate the heart beat during incubation as a survival sign. On the desired day, each well was fixed with 4% PFA and prepared for whole mount IHC.

For neuronal tracing and labeling, we used the FAST DiI crystal (FAST DiI™ solid; DiIΔ9,12-C18(3), CBS (1,1'-Dilinoleyl-3,3,3',3'-Tetramethylindocarbocyanine, 4-Chlorobenzenesulfonate, D7756, Fisher Scientific), which is weakly fluorescent in water but highly fluorescent and photo stable when incorporated into the cell membranes. Briefly, FAST DiI was inserted to the mesencephalon at E9 using a sharp-ended needle. After insertion of FAST Dil, images were captured by stereomicroscope to monitor the location of DiI (at DIV 0). After placing the embryos into 24-well plates in culture medium, embryos were monitored every 6 hrs and fixed with 4% PFA on the desired day, then whole mount IHC with NAA was

performed to have a better view of the neural fibers and followed with sectioning and imaging of the DiI positive cells in cerebellum.

In situ hybridization (ISH)

All of the ISH experiments were carried out on the E12 CD1 mice using RNAscope ACD HybEZ™ II Hybridization System and RNAscope® Multiplex Fluorscent Reagent Kit v2 (Advanced Cell Diagnostics, Hayward, CA, USA). Briefly, embryos were fixed in 10% (vol/vol) neutral buffered formalin at room temperature for 24 h, dehydrated, and embedded in paraffin. Tissue sections cut at 10 μm thickness were processed for RNA *in situ* detection according to the manufacturer's user manual. Sequences of the probes used in the study are as follows: Mm-Fgf8 (313411, ACD), Mm-Wnt1-C2 (401091-C2, ACD), Mm-Otx2-C3 (444381, ACD). Flourophores (TSA® Plus, Perkin Elmers, Waltham, MA, USA)) used in the study are as follows: Fluorescein (NEL741E001KT), Cyanine 3 (NEL744E001KT), and Cyanine 5 (NEL745E001KT).

Western blotting analyses

Equal amount of proteins were separated by SDS/PAGE in 10–15% precast gels (Bio-Rad, Hercules, CA, USA) and transferred onto the PVDF-membrane. For the Western blot analysis, membranes were blocked in 5% nonfat dry milk (NFDM) in TBS containing 0.02% Tween 20 (TBST) and then incubated overnight at 4°C with primary antibodies as follows: α-synuclein (sc-69977, Santa Cruz) 1:2000, p75NTR (8238, Cell Signaling) 1:1000, Caspase 3 (9665, Cell Signaling)1:1000, and Cleaved Caspase 3 (9664, Cell Signaling) 1:1000. Secondary antibodies as follows: HRP conjugated goat anti-mouse IgG (AP308P, Millipore) 1:6000 and

HRP conjugated goat anti-rabbit IgG (12-348, Millipore) 1:6000. Binding was assessed using the Enhanced Chemiluminescence (ECL) protocol on Scientific Imaging Film.

Imaging and figure preparation

For bright field microscopy, images were captured using Zeiss Axio Imager M2 microscope (Zeiss, Toronto, ON, Canada). Images were then analyzed with a Zeiss Microscope Software (Zen Image Analyses software) (Zeiss, Toronto, ON, Canada). For fluorescence microscopy of the entire cerebellum sections, a Zeiss Lumar V12 Fluorescence stereomicroscope (Zeiss, Toronto, ON, Canada) equipped with a camera was used to capture the images. Images were then analyzed using Zen software. For high magnification fluorescence microscopy, a Ziess Z1 and Z2 Imager and a Zeiss LSM 700 confocal microscope (Zeiss, Toronto, ON, Canada) equipped with camera and Zen software were used to capture and analyze images. Images were cropped, corrected for brightness and contrast, and assembled into montages using Adobe Photoshop CS5 Version 12.

Statistical analysis

Otx2 positive cell count data are presented as the mean ± standard error of mean (SEM) and they were analyzed using One-Way ANOVA test followed by Tukey`s multiple comparison test. We considered $p > 0.05$ as non-significant result. All statistical analysis was performed using GraphPad Prism 6 software for windows.

3.3 Result

The early subset of CN neurons at the rostral end of the cerebellar primordium are derived from the mesencephalon

In mouse embryos, at the rostral end of the cerebellar primordium a few cells are immunopositive for neuronal marker such as neurofilament-associated antigen (NAA) 3A10 (Marzban et al., 2008a) at the early stage of E9 (Fig. 3.1A-D). To explore whether these cells cross the isthmus, we used anti-α-synuclein (SNCA) antibody, which is expressed in subset of CN neurons (Zhong et al., 2010b). In a sagittal section of the E9 cerebellar primordium, we found that $SNCA^{+}$ neurons run from the dorsal mesencephalon, cross the isthmus, and pass in through the rostral end of the NTZ (Fig. 3.1E-F).

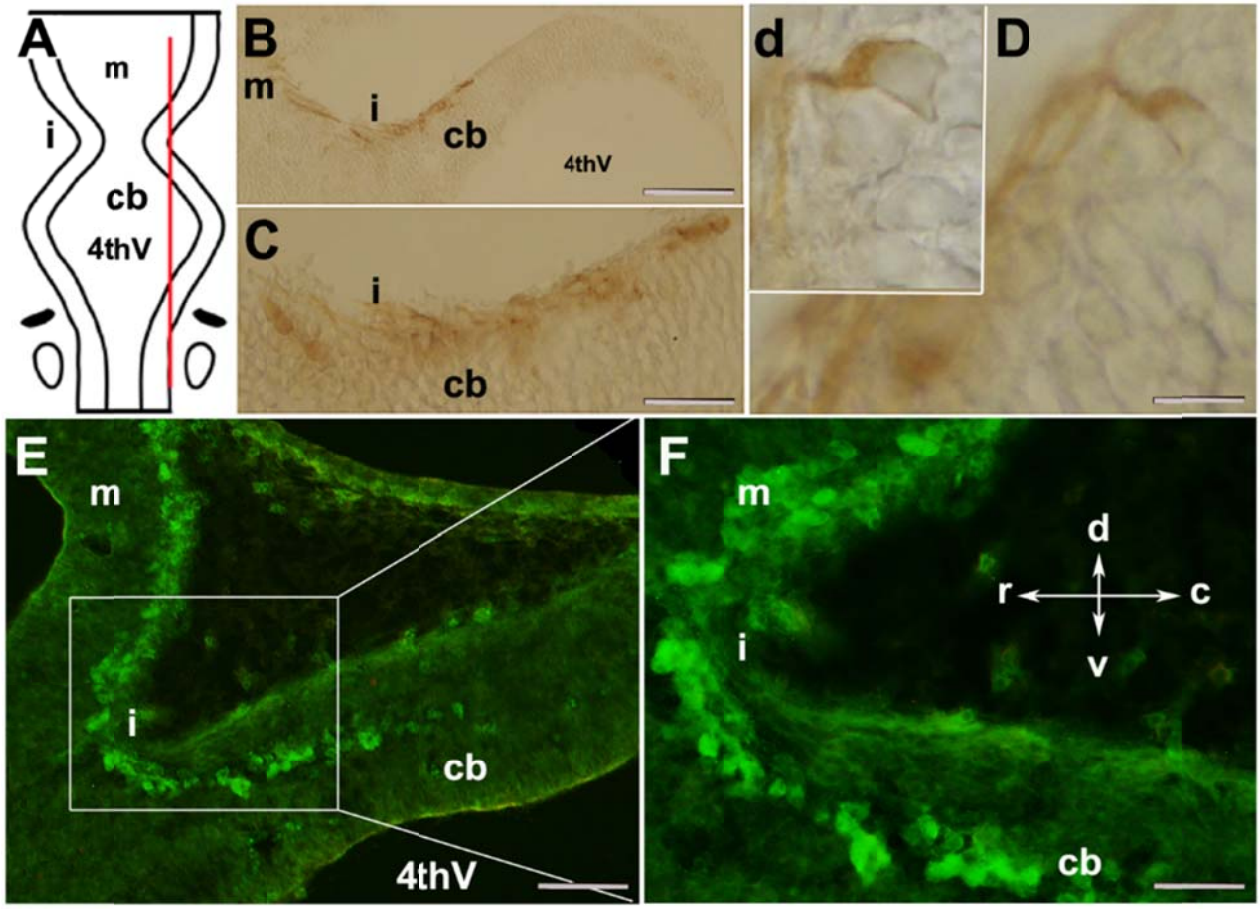

Figure 3. 1 Cerebellar primordium immunolabeled with NAA and SNCA shows that a subset of neurons is present at the rostral end of cerebellum at E9

A. Dorsal view of the schematic illustration of the cerebellar primordium, mesencephalon, isthmus, and 4th ventricle. The red line shows the sagittal plane about which the section shown in B–D was taken.

B-D. Sagittal section through the cerebellar primordium at early stage E9. Immunoperoxidase-labeled with NAA 3A10 shows the presence of neurons in the cerebellar primordium that crosses the isthmus (i) and continues to the mesencephalon. **C.** A higher magnification of **B**. **D.** Differentiated neurons at E9 are visible; a higher magnification is shown in the inset, **d**.

E–F. Sagittal section through the cerebellar primordium at late E9. Immunofluorescence labeling of SNCA shows $SNCA^+$ (green) CN neurons in the mesencephalon, at the isthmus (i) and in the rostral part of cerebellar primordium (cb). **F.** A higher magnification of **E**.

Abbreviations: 4thv, 4th ventricle; cb, cerebellum; i, isthmus; m, mesencephalon; r, rostral; c, caudal; d, dorsal; v, ventral

Scale bar, 100 μm in B; 50 μm in C, E; and 20 μm in D

To determine the position of the $SNCA^+$ cells in the NTZ compared to the rhombic lip-derived $Lmx1a^+$ CN neurons (E10-12) (Fink et al., 2006a), double immunocytochemistry of SNCA and Lmx1a was performed on sagittal sections of E12 cerebellar primordium (Fig. 3.2 A-D, medial section and Fig. 3.2 E-H, lateral section). This shows that an $Lmx1a^+$ population of CN neurons, which extends from the rhombic lip, flanks $SNCA^+$ neurons in the NTZ that continues rostrally to the mesencephalon (Fig. 3.2A, E). The majority of neurons are not

SNCA+/Lmx1a^{+} but some of the neurons are co-labeled, and the latter may be because the cells are overlapped (Fig. 3.2 D, H).

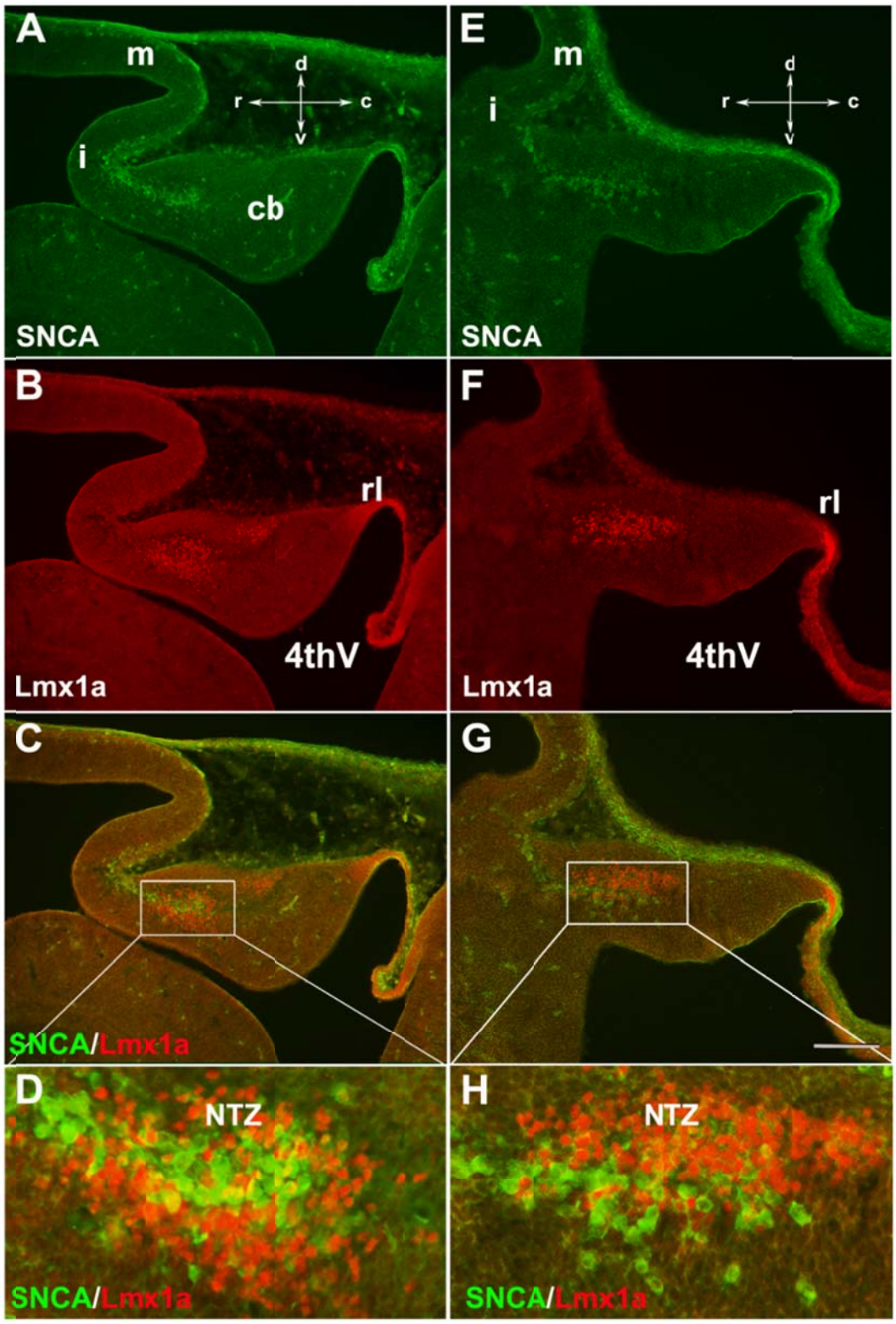

Figure 3. 2 Sagittal section through the cerebellar primordium at E12 double immunofluorescence labeled with SNCA and Lmx1a at medial (A–D) and lateral (E–H) sections **A–G**. SNCA (green, A and E) and Lmx1a (red, B and F) immunopositive cells are located at a CN neuron temporary site called the NTZ. The $SNCA^+$ cells continue to the mesencephalon and $Lmx1a^+$ cells continue to the rhombic lip. Merged images show that the $SNCA^+$ cells form a population of CN neurons distinct from the rhombic lip-derived cells ($Lmx1a^+$) in NTZ (C and G). D and H show a higher magnification of C and G, respectively.

Abbreviations: 4thV, 4th ventricle; cb, cerebellum; i, isthmus; m, mesencephalon; rl, rhombic lip; r, rostral; c, caudal; d, dorsal; v, ventral

Scale bar, 100 μm

To explore the possibility that the $SNCA^+$ cells originate from the mesencephalon, Otx2 IHC was performed during early cerebellar development. *Otx2* is highly expressed in the mesencephalon and its caudal limit is the boundary with the metencephalon (i.e. the isthmus) (Kurokawa et al., 2004). However, IHC at the sagittal section of E12 cerebellum showed that a subset of $Otx2^+$ cells from the mesencephalon continue caudally, cross the isthmus and end in the rostral region of the cerebellar primordium (Fig. 3.3 A-B, medial) and Fig 3.3 C-D, lateral).

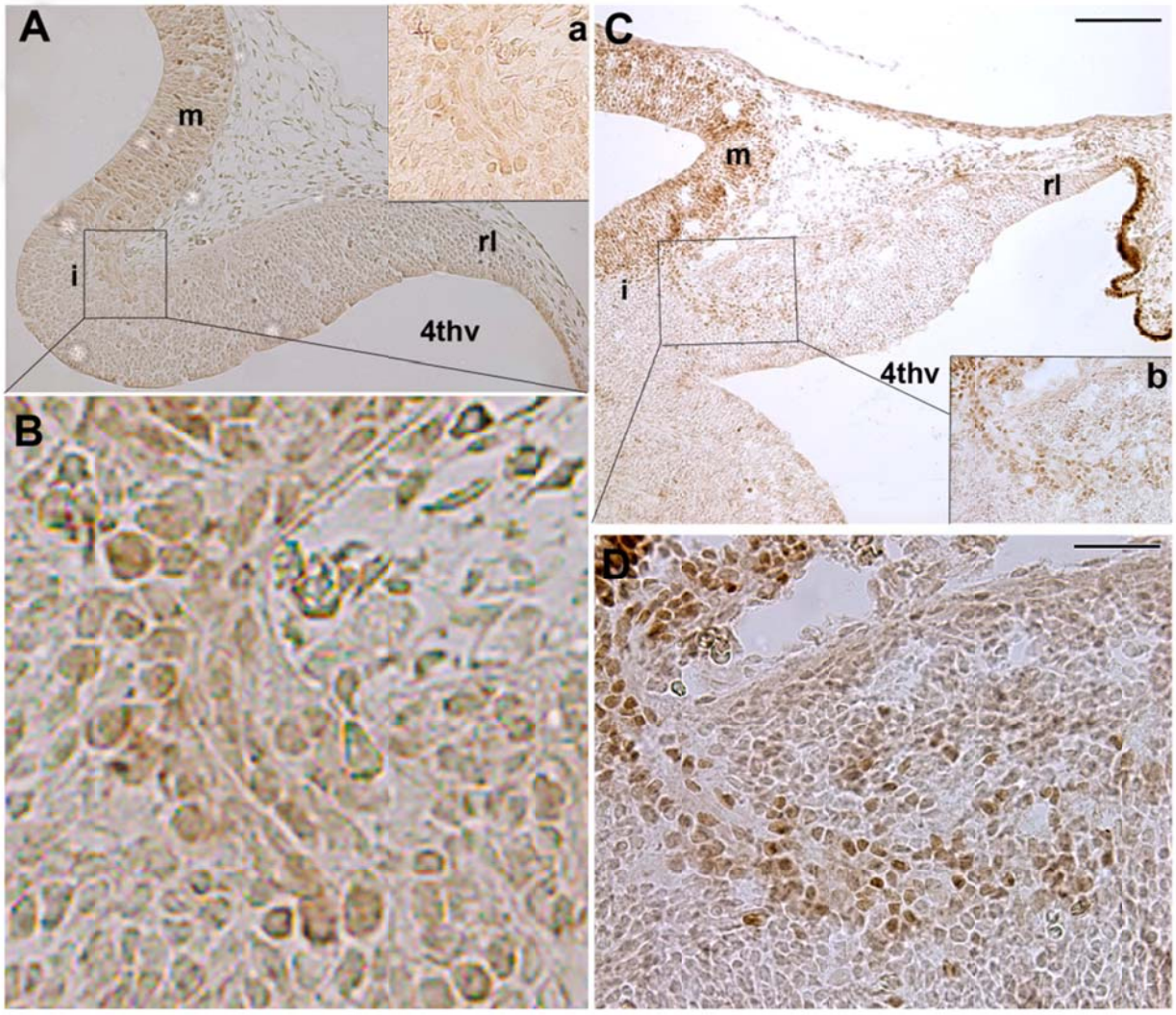

Figure 3. 3 Sagittal section through the cerebellar primordium at E 12, peroxidase immunolabeled by Otx2

A–D. Sagittal section through medial (A) and lateral (C) cerebellar primordium show high Otx2 immunoreactivity at the mesencephalon and a few $Otx2^{+}$ cells cross the isthmus and position at the rostral part of cerebellar primordium at the NTZ. The boxed regions in **A** (a) and **C** (b) are shown at higher magnification in **B** and **D**, respectively.

Abbreviations: 4thv, 4th ventricle; i, isthmus; m, mesencephalon; rl, rhombic lip

Scale bar, 200 μm in A, C; 50 μm in D, B

To understand whether the Otx2^{+} cells that continue to the NTZ are SNCA^{+}, double IHC with Otx2 and SNCA was performed in E12 sagittal sections. Otx2 is highly expressed in the mesencephalon (Fig. 3.4A, B), and was co-expressed with SNCA^{+} cells in the NTZ (Fig. 3.4C-E). Primary dissociation of cerebellar culture at E10 and DIV 4 showed that SNCA^{+} cells were co-expressed with Otx2 (Fig. 3.4 F–H).

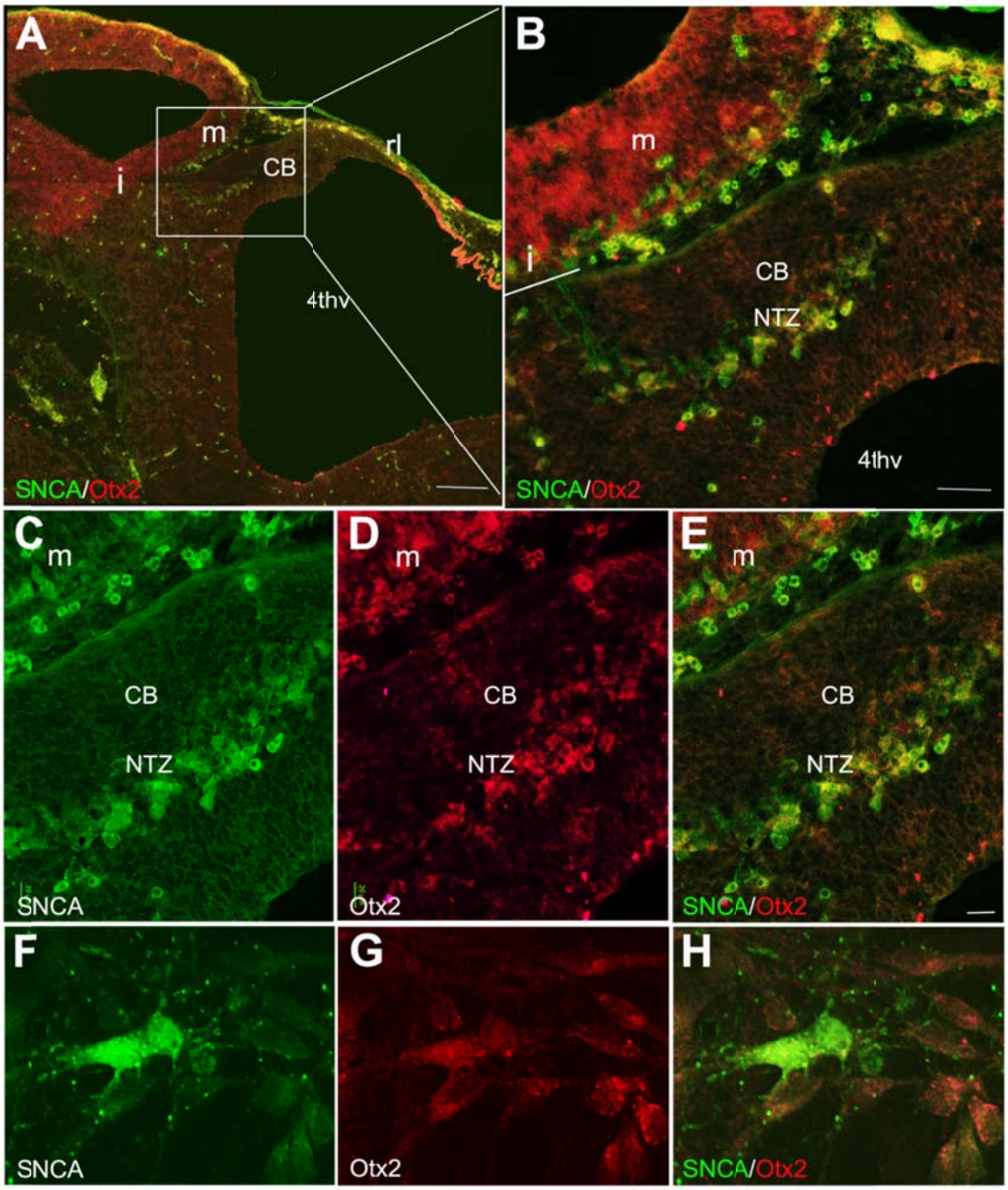

Figure 3. 4 Sagittal section through the cerebellar primordium at E12 double immunofluorescence labeled with SNCA and Otx2 in section and primary cerebellar culture

A–E. Double immunolabeling of SNCA (green) and Otx2 (red) on the sagittal section of the cerebellar primordium at E12. **A**. High Otx2 immunoreactivity in the mesencephalon. $SNCA^+$ cells in the mesencephalon accompany Otx2 $^+$ cells cross the isthmus (i) and enter the NTZ. **B**. higher magnification of **A**. **C-E**. Immunolabeling with SNCA (green, **C**) and Otx2 (red, **D**) and merged (**E**) shows co-expression in the NTZ.

F–H. Primary dissociated cerebellar culture obtained at E10, DIV 4 shows co-expression of $SNCA^+$ cells with Otx2.

Abbreviations: 4thv, 4th ventricle; CB, cerebellum; i, isthmus; m, mesencephalon; rl, rhombic lip; NTZ, nuclear transitory zone

Scale bar, 200 μm in A; 50 μm in B, F, G, H; 20 μm in C, D, E

To study the presence of Otx2 positive cells and count them during early and mid-embryonic stages; slides with the sections of E12, 13, 14 and 15 were labeled for Otx2 (Fig. 3.5 A-D, and a-d). And shown Otx2 positive cells are still present in the cerebellar primordium from E12 to E15. The total amount of Otx2 positive cells in cerebellar primordium were counted in slides from E12 to E15 and compared (Fig. 3.6). The comparison between these embryonic days indicates that there is a slight increase in the number of Otx2 positive cells between E12-E15. There is a significant difference between E12 and E14 (**,P<0.01) and E15 (***,P<0.001). Also there is a significant difference between E13 and E15 (#, P<0.05).

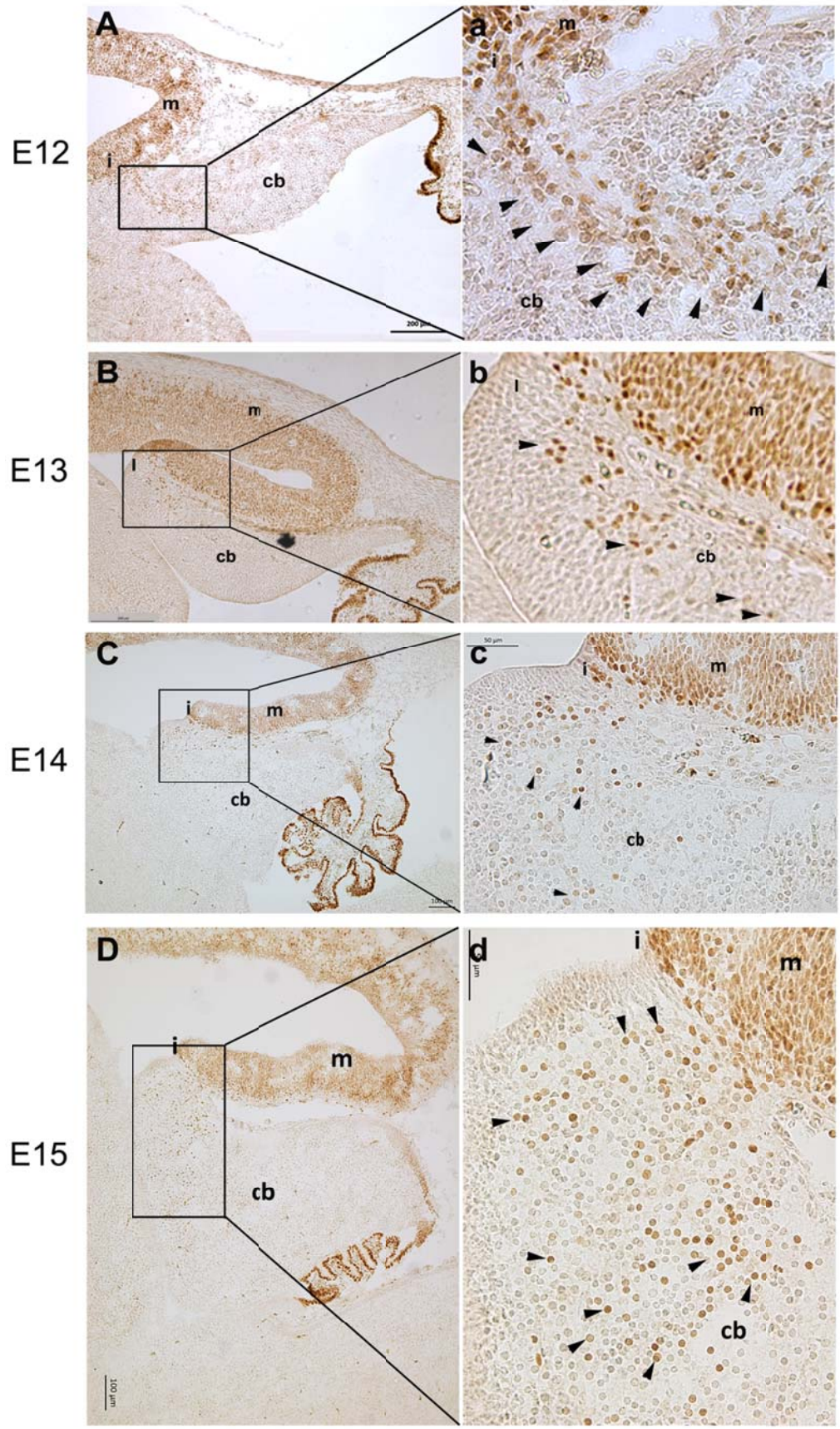
E12
E13
E14
E15
A
a
m
i
cb
B
b
l
C
c
D
d
200 µm
100 µm

Figure 3. 5 Sagittal sections through the cerebellar primordium at E 12, 13, 14 and 15 peroxidase immunolabeled by Otx2

A–D. Sagittal section through the cerebellar primordium (E12-E15) shows high Otx2 immunoreactivity at the mesencephalon and a few $Otx2^+$ cells cross the isthmus and position at the rostral part of the cerebellar primordium at the NTZ. The boxed regions in **(A-D)** are shown at higher magnification in **(a-d)** respectively.

Abbreviations: i, isthmus; m, mesencephalon; cb, cerebellum

Scale bar, 100 μm in A-D; 50 μm in a-d

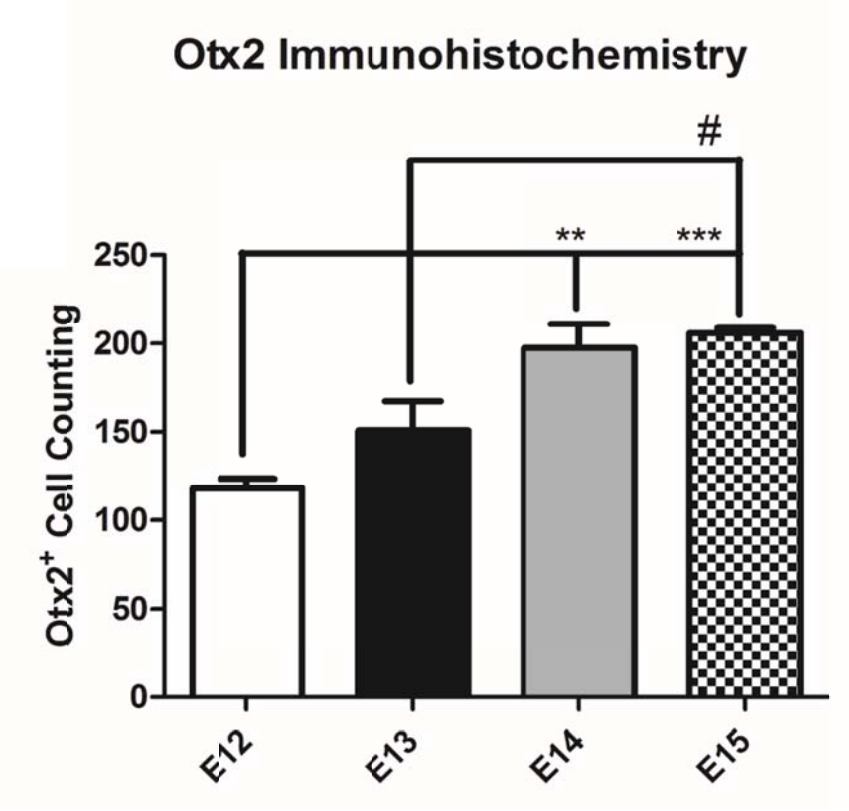

Figure 3. 6 Comparison of Otx2 positive cell counting between E12 to E15 cerebellar primordium

The comparison between these embryonic days indicates that there is a slight increase in the number of Otx2 positive cells between E12-E15. There is a significant difference between E12 and E14 (**,P<0.01) and E15 (***,P<0.001). Also there is a significant difference between E13 and E15 (#, P<0.05). This study was analyzed by One-Way ANOVA test followed by Tukey`s multiple comparison test.

To determine the expression patterns of *Otx2*, *Wnt1*, and *Fgf8* in cerebellar primordium at E12, we employed highly-sensitive RNAscope ISH, which allowed us to simultaneously detect three probes. *Otx2* mRNA signal is highly expressed in the mesencephalon and extend caudally cross the isthmus to the rostral cerebellar primordium in NTZ (Fig. 3.7 D). *Wnt1* mRNA signal is high in the mesencephalon at isthmus organizer and similar to *Otx2*$^{+}$ cells, extend to the rostral cerebellar primordium in NTZ (Fig. 3.7 E). *Fgf8* mRNA signal is present in few scattered cells in rostral end of the cerebellar primordium (Fig. 3.7 F). *Otx2*$^{+}$ cells at NTZ are co-expressed with Wnt1^{+} cells (Fig. 3.7 A, B and G).

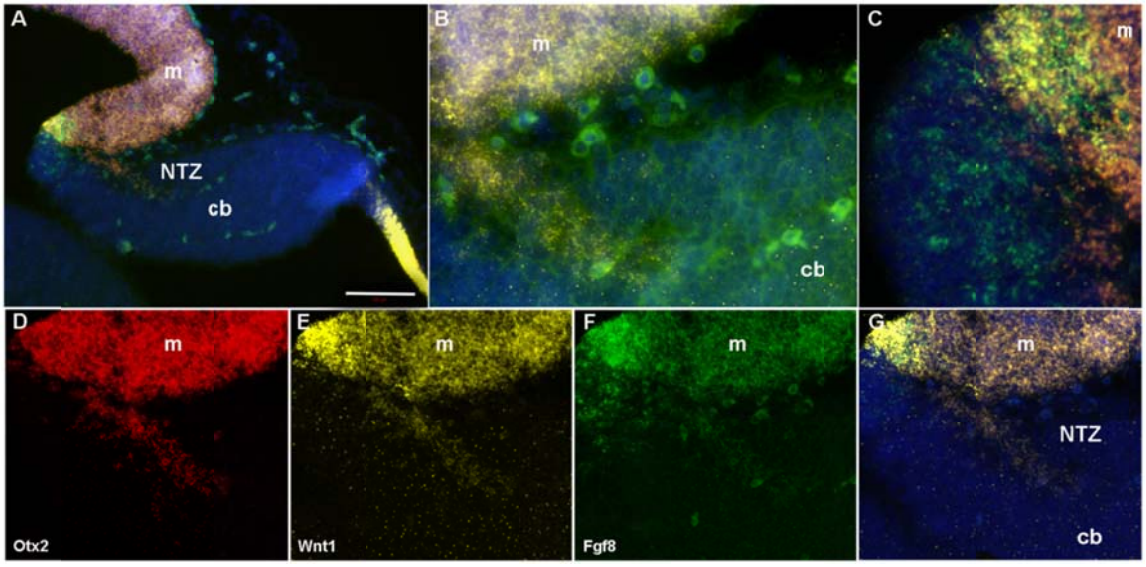

Figure 3. 7 Expression patterns of *Otx2*, *Wnt1*, and *Fgf8* ligands at E12 mouse cerebellum evaluated by RNA *in situ* hybridization (RNAscope). In situ hybridization of all ligands (Otx2 red, Wnt1 yellow, and Fgf8 green) on embryo sections from CD1 mice.

A-C) Merged channels of the *in situ* hybridization of all ligands; *Otx2*, *Wnt1*, and *Fgf8* mRNA probe at low (A) and high (B) magnification, and image captured by confocal microscopy (C).

D) *Otx2* mRNA signal is strong in the mesencephalon and extend as tail to the rostral cerebellar primordium in NTZ.

E) *Wnt1* mRNA signal is highest in the mesencephalon and extend to isthmus and seen like a tail in the rostral cerebellar primordium in NTZ.

F) *Fgf8* mRNA signal is present in scattered cells at the rostral cerebellar primordium in NTZ.

G) Panels D, E, F are merged in (G) and blue signal is labeling with DAPI.

Abbreviation; cb, cerebellum; m, mesencephalon; NTZ, nuclear transitory zone

Scale bar: 100 μm in A, 20 μm in B, C; 50 μm in D-G

The results of IHC and ISH experiment suggest that the subset of cerebellar nuclei neurons is derived from the mesencephalon. Thus, we used FAST Dil to further investigate cell migration. We applied FAST DiI with a sharp-ended needle in dorsum of caudal mesencephalon region. After 4 days *in vitro*, mesencephalic-derived cells which are labeled with DiI clearly present and migrate in both rostrally and caudally directions. To eliminate the number of cell population exposed to DiI at different wave of generation in mesecephalon during the period experiment, we limited DiI exposure for only 24 hrs. (Fig. 3.8). It is reported that the earliest neuronal population are in mesencephalon and project caudally (Stainier and Gilbert, 1990) . To determine whether early generation of mesencephalic cells migrate to the cerebellar primordium,

we removed the inserted DiI after 24 hrs. To our surprise, almost all DiI+ cells were migrated caudally toward the cerebellar primordium, not rostrally (Fig. 3.9).

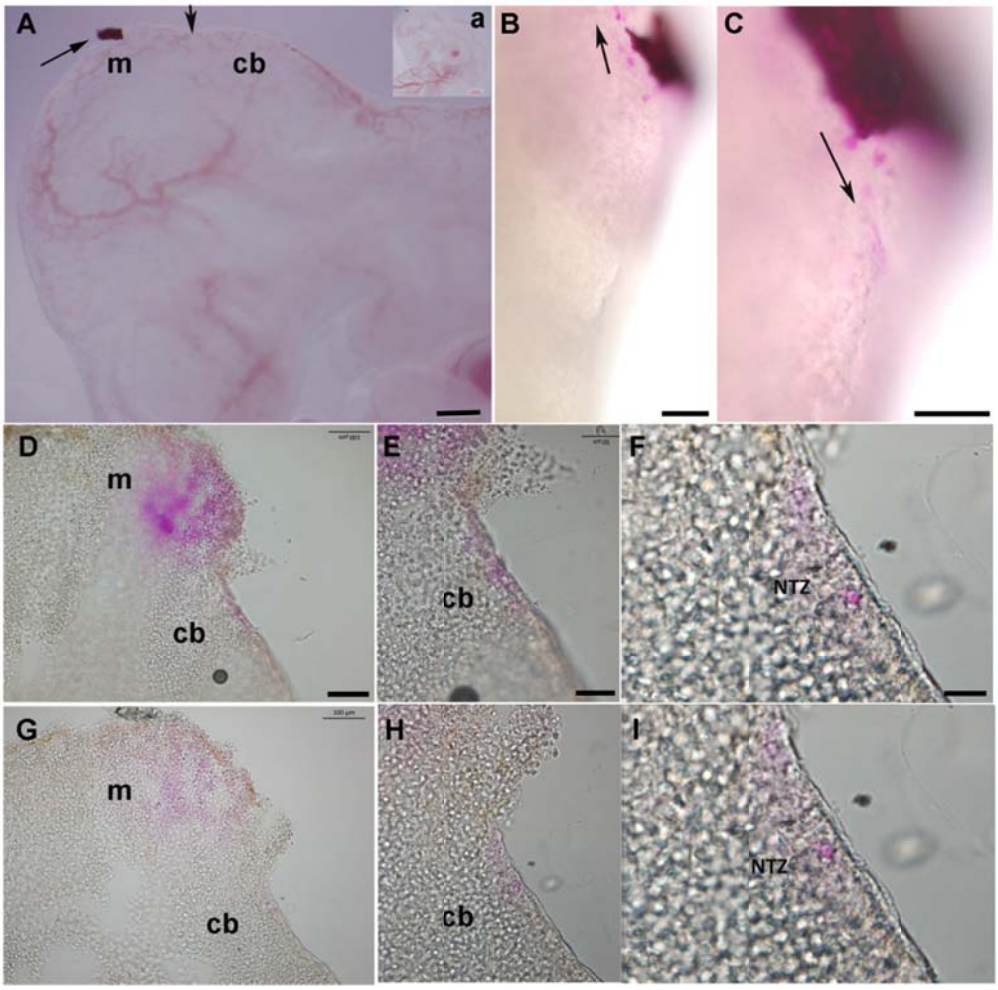

Figure 3. 8 Fast DiI applied to embryo at E9 and maintained in place for 4 days in vitro (DIV 4).

A, a. Fast DiI inserted in mesencephalon at E9 (DIV 0), arrow shows inserted location of DiI crystal in mesencephalon and arrowhead indicate isthmus.

B-C. DiI positive cells directed rostral to mesencephalon (B) and caudally to rostral cerebellar primordium (C).

D-F. low and high magnification shows few cells in the rostral cerebellar primordium in NTZ at level of medial cerebellar section.

G-I. low and high magnification shows few cells in the rostral cerebellar primordium in NTZ

at level of lateral cerebellar section.

Abbreviations: cb, cerebellum; m, mesencephalon; NTZ, nuclear transitory zone

Scale bar: 500 µm in a; 200 µm in A; 100 µm in B-D, and G; 50 µm in E, H; 20 µm in F,I

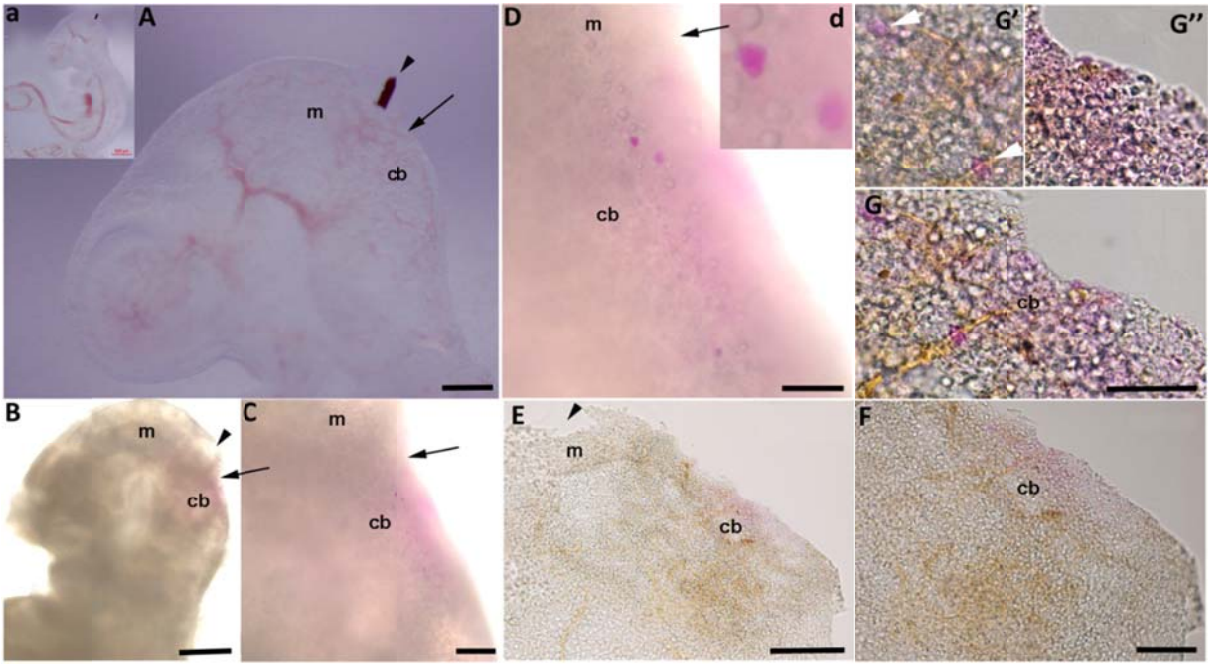

Figure 3. 9 Fast DiI applied to embryo at E9 and removed after 24 hrs.

A, a. Fast DiI inserted in mesencephalon at E9 (DIV 0) (indicated by arrowhead) and arrow shows the isthmus.

B. DiI positive cells present in cerebellar primordium after DIV 6.

C-D, d. A higher magnification from the caudal to mesencephalon and rostral rhombencephalon shows DiI positive cells in cerebellar primordium

E-G. low and high magnification shows clearly cells with DiI labeling in the rostral cerebellar primordium in NTZ after whole mount IHC with NAA and sectioning.

Abbreviations: cb, cerebellum; m, mesencephalon

Scale bar: 500 µm in a, B; 250 µm in A; 200 µm in E; 100 µm in C, D,F; 50 µm in G.

P75 neurotrophin receptors are expressed in the NTZ

The mesencephalic neural crest cells during the migratory phase express *Otx2* gene (Kimura et al., 1997). At the embryonic stage, neural crest stem cells (NCSCs) arise from the transient neural crest and create tissues that initially generate neural crest stem cells, in which migrating cells express the early neural crest marker, p75ntr (protein 75 the neurotrophin receptor) (Jiang et al., 2008). Neuronal proliferation/differentiation and the neuronal fiber formation are regulated by p75ntr, which is mainly expressed during early development (Bernabeu and Longo, 2010a, Dechant and Barde, 2002b). The p75ntr (a typical neural crest cell marker) (Lee et al., 2002, Schwarz et al., 2008) was used to test for $SNCA^{+}/Otx2^{+}$ cells that arise probably from the neural crest population.

To determine whether $SNCA^{+}/Otx2^{+}$ neurons are p75ntr immunopositive, double immunolabeling with SNCA was performed (Otx2 and p75ntr are both rabbit polyclonal antibody and it is not possible to do double immunolabeling) . Section immunolabeling revealed that $SNCA^{+}$ cells in the NTZ were co-labeled with p75ntr that is localized in the cell membrane (Fig. 3.10A–C, H). This data was further confirmed by Western blot analysis of SNCA and p75ntr expression during different embryonic days, E11, E13 and E15 (Fig. 3.10h). In addition, in primary dissociated cerebellar culture at E10, DIV 3 showed that p75ntr is expressed with $SNCA^{+}$ neuronal cell membrane (with punctate appearance, Fig. 3-10 D–G (Dechant and Barde, 2002b)). P75ntr and TrkA (which also labels neural crest-derived neurons) (Tomellini et al., 2014, Young et al., 1999, Martin-Zanca et al., 1990, Harris and Erickson, 2007) are two distinct nerve growth factor receptors that probably form a ternary TrkA/NGF/p75 complex (Wehrman et al., 2007). To determine whether TrkA is expressed in a subset of neurons in the NTZ, we have

performed double immunolabeling with NAA and showed TrkA and NAA co-expression in the NTZ (Fig. 3.10 I–K).

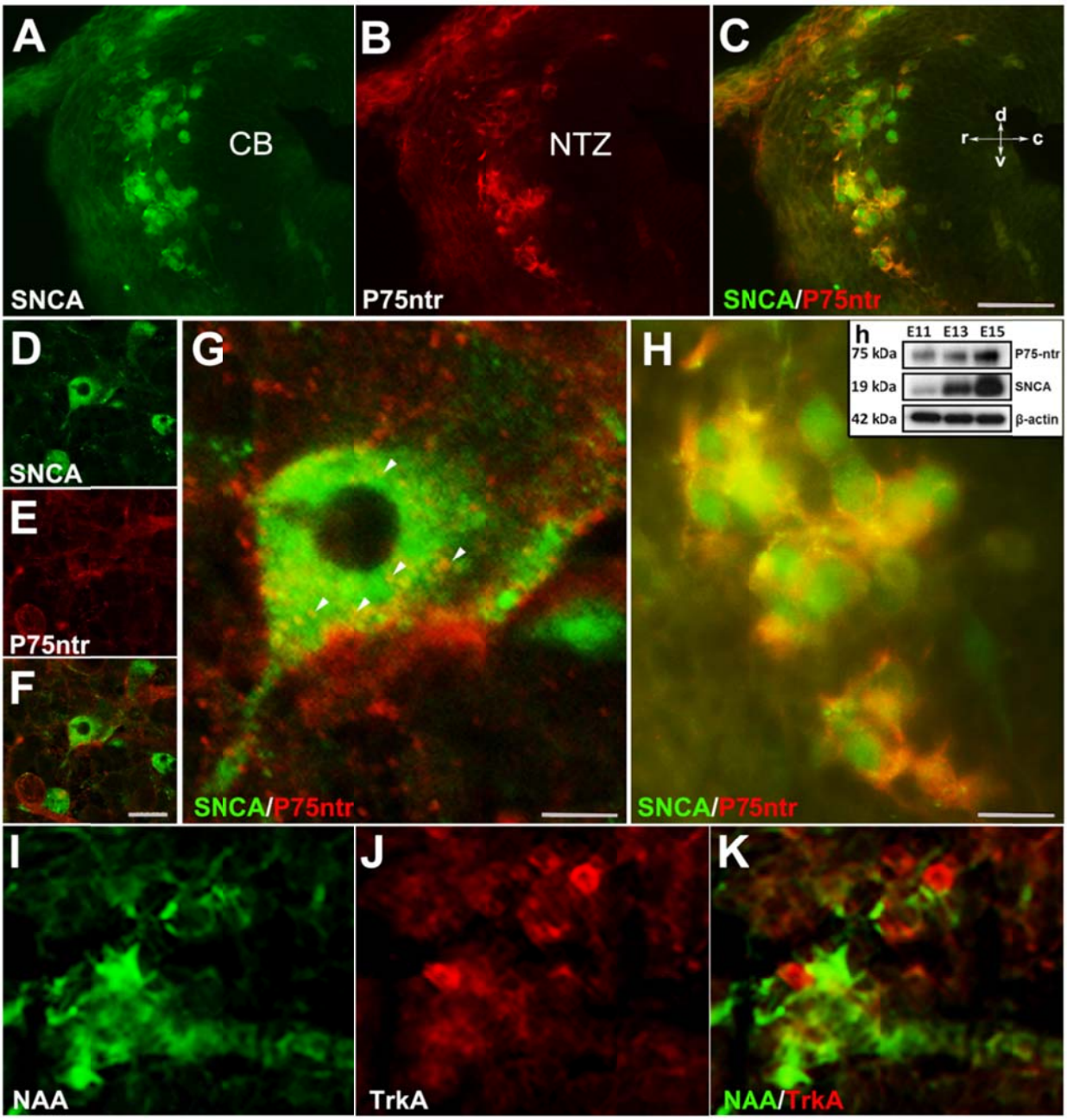

Figure 3. 10 Sagittal section through cerebellar primordium at E10, double immunofluorescence labeled with SNCA and 75ntr

A–C, H. Double immunofluorescence labeled with SNCA (**A**, green) and P75ntr (**B**, red) show co-labeled cells (**C**, merged) in the NTZ and a higher magnification for NTZ is shown in H.

h. Western blotting of SNCA and P75ntr expression during cerebellar development.

Immunoblots of total cerebella lysate from embryos at different embryonic days, E11, E13 and E15 indicate an increase in expression of SNCA and P75ntr from E11 to E15. Protein loading was confirmed using βactin.

D–G. Primary dissociated cerebellar culture obtained from E10 mouse embryo, DIV 3, double immunofluorescence labeled for SNCA (**D**: green) and P75ntr (**E**: red) and merged (**F**). **G** is a higher magnification of **F**; punctuate immunoreactivity of $SNCA^{+}$ cells is marked with arrowheads.

I-K. Double immunofluorescence labeling of the E10 sagittal section for NAA 3A10 (green) and TrkA (red) shows TrkA immunoreactive cells located in the NTZ.

Abbreviations: cb, cerebellum; NTZ, nuclear transitory zone

Scale bar, 50 μm in A–C and D–F; 20 μm in H and 10 μm in G

Immunolabeling with NAA 3A10 showed that a set of the neurons and neuronal processes are present in the NTZ in the cerebellar primordium core at E12 (Fig. 3.11 A). To determine whether the NAA $3A10^{+}$ combination of neurons and neuronal processes at the core of cerebellar primordium express p75ntr, double immunolabeling was performed (Fig. 3.11 A–C, H). The results showed that the $NAA^{+}/p75ntr^{+}$ combination of neurons and neuronal processes from the isthmus continue to the NTZ and terminate on the subpial surface at the midpoint of the rostrocaudal cerebellar primordium (Fig. 3.11 H). In dissociated embryonic cerebellar culture at

E10, DIV 21 showed that p75ntr immunoreactivity is localized in the membrane of NAA^{+} neuronal somata and process (Fig. 3.11 D–G).

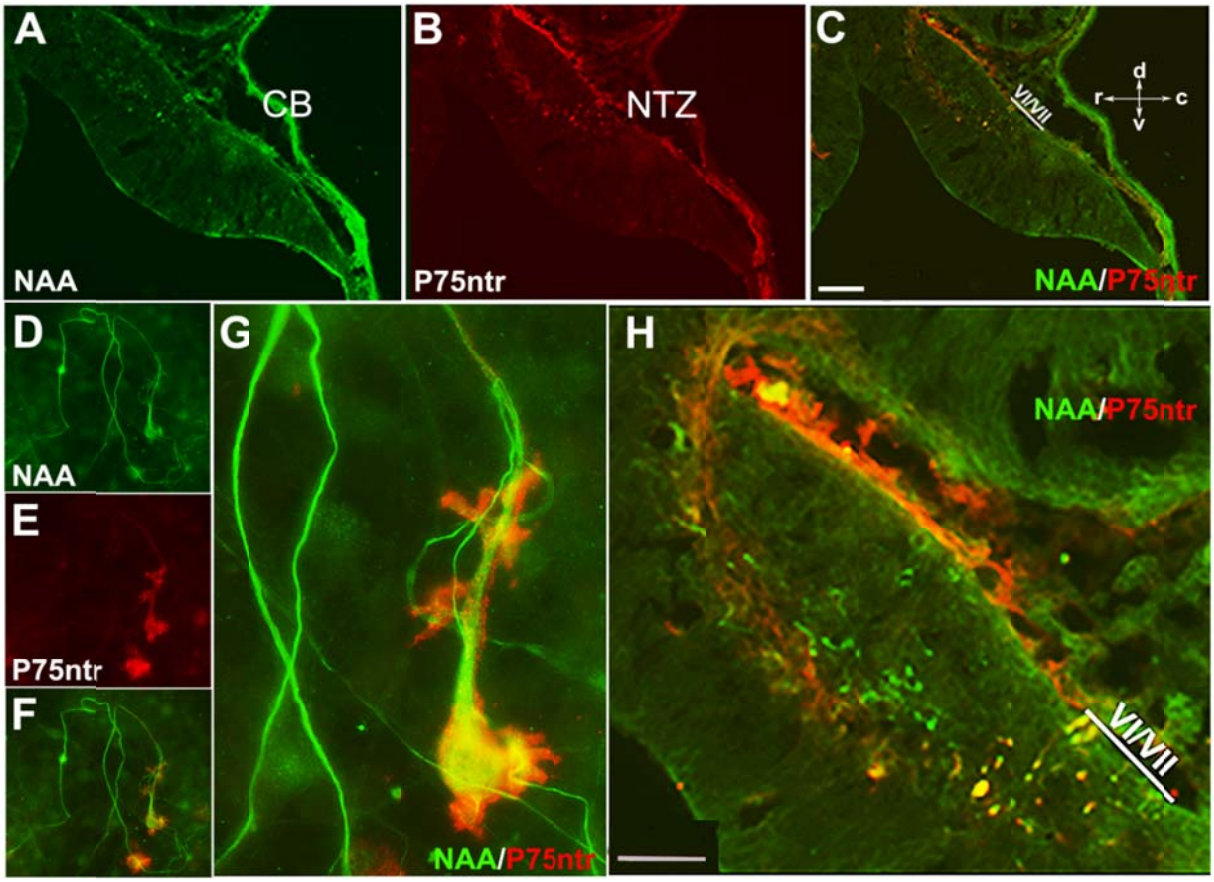

Figure 3. 11 Sagittal section through cerebellar primordium at E12, double immunofluorescence labeled with NAA 3A10 and P75ntr (we used NAA to determine whether P75ntr fibers are nerve fibers)

A–C, **H.** Double immunofluorescence labeled with NAA 3A10 (**A**; green) and P75ntr (**B**: red) show co-labeled cells and fibers (**C**; merged) in the NTZ and a higher magnification, shown in **H**, indicates the termination of fibers to the subpial surface in rostrocaudal midpoint of the cerebellar primordium.

D–G. Primary dissociated cerebellar culture at E10 mice embryo, after 21 days in vitro (DIV 21), double immunofluorescence labeled for NAA (green) and P75ntr (red). Immunopositive

neuronal somata and axons shown with NAA 3A10 (**D**), while P75ntr (**E**) immunoreaction is localized in cell membrane that are merged in **F**. **G**. A higher magnification of panel **F**.

Abbreviations: cb= cerebellum, NTZ= nuclear transitory zone

Scale bar, 100 µm in A–C; 50 µm in H and 20 µm in D–F

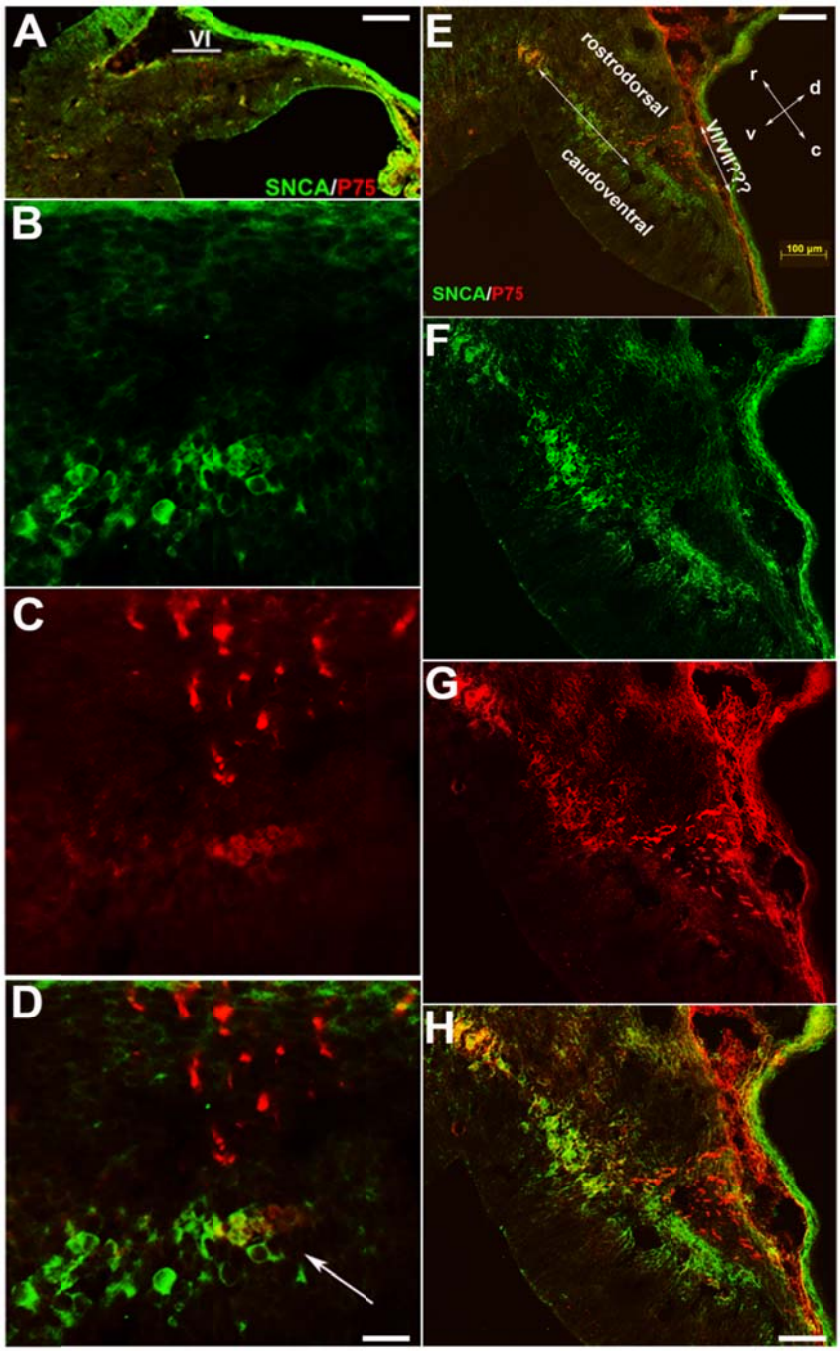

Figure 3. 12 Double immunolabeling with SNCA and P75ntr at E10 and E12 shows a combination of neurons and fibers present at the core of cerebellar primordium

A–D. Double immunolabeling with SNCA (green) and P75ntr (red) at E10 shows more co-labelled cells at the caudal end of the NTZ (**D**, merged; arrow) that continue with fibers that terminate on the subpial surface at the rostrocaudal midpoint of the cerebellar primordium.

E–H. Double immunolabeling with SNCA (green) and P75ntr (red) at E12 shows more co-labelled cells at the rostral end of the NTZ (**H**, merged).

Scale bar, 100 μm in A,E; 50 μm in B–D, F–H, I; 20 μm in J–L

Double immunofluorescence labeling of p75ntr with SNCA at E10 showed that expression is mostly localized in neurons at the caudal end (Fig. 3.12 A–D), while at E12, expression is also localized in the neurons at the rostral end of the NTZ (Fig. 3.12 E–H). $P75ntr^{+}/SNCA^{+}$ neurons and neuronal processes combine at the cerebellar primordium core and terminate on the subapical surface at the rostrocaudal midway point of the cerebellar primordium, probably at putative lobules VI/VII (Fig. 3.12 A–H).

Fate of the subset of CN neurons beside transient expression of SNCA

To understand the fate of SNCA and SNCA+ cells in a subset of CN neurons, the following experiments were performed. First, we determined the pattern of SNCA protein expression in the rostral cerebellar primordium from E9 was most prominent in the CN in NTZ (Figs 3.1 and 3.2). At E13, SNCA expression was weak in neuronal cell body (Fig. 3.13 A, B, b). At E15/16, SNCA expression disappeared from the CN neuronal cell bodies (Zhong et al., 2010b) (data not shown). Around the perinatal cerebellum, SNCA expression was localized to

the axonal terminal field and there was no SNCA expression in any cell body on the cerebellar cortex and CN (Fig. 3.13 C–E).

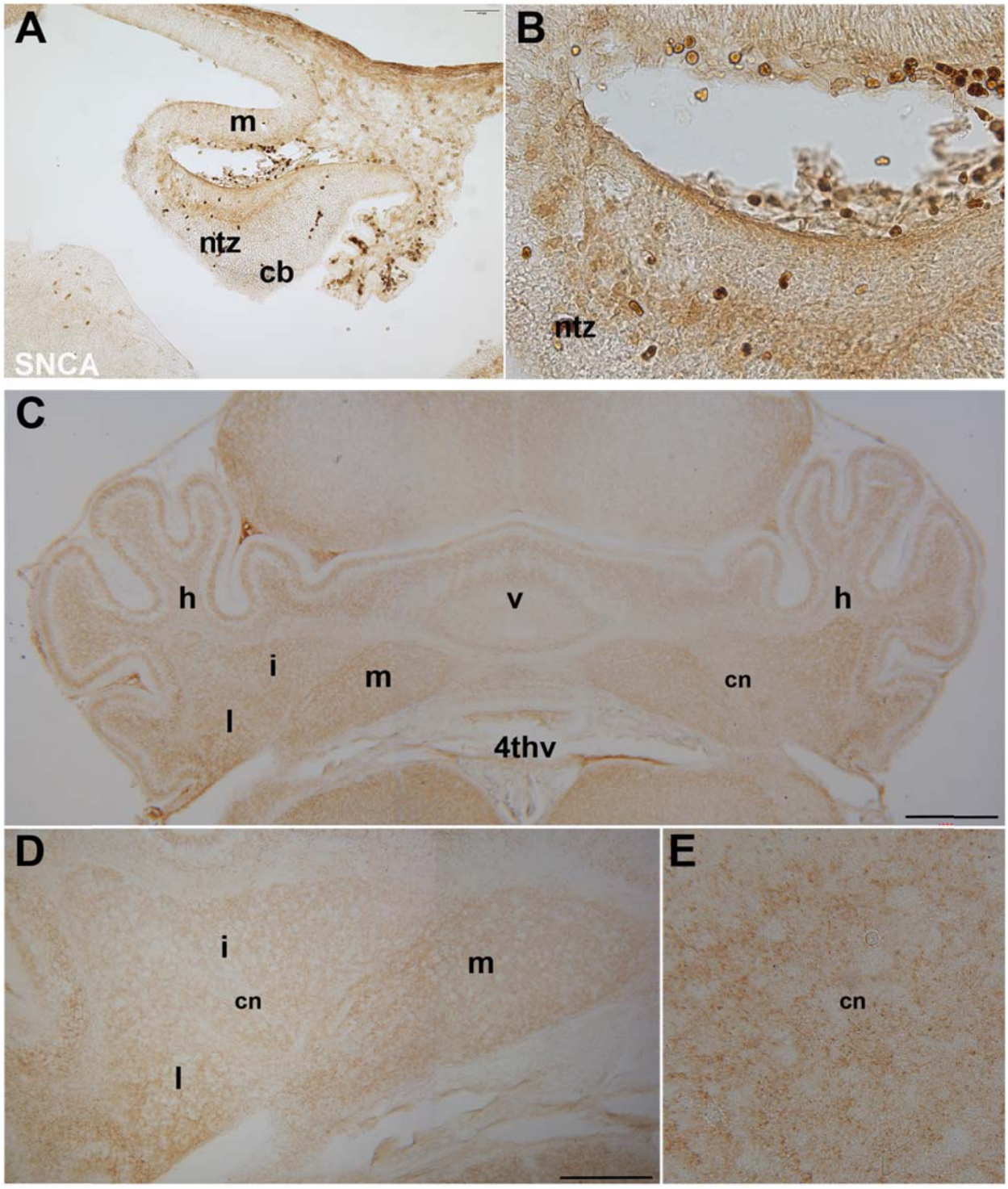

Figure 3. 13 SNCA peroxidase immunolabeling at E13 and P4

A. Sagittal section of cerebellar primordium at E13 immunolabeled with SNCA shown weak SNCA expression in somata.

B. are higher magnification for A.

C. A frontal section of the cerebellum at P4 with SNCA immunoperoxidase labeling shows that there is no immunoreactivity in the cell body in all three cerebellar nuclei (CN); medial (m), interposed (i) and lateral (l), but it is present distinctly in the axon terminals.

D, E. are higher magnification for C.

Abbreviations: cn, cerebellar nuclei; h, hemisphere; i, interposed nucleus; l, lateral nucleus; m, medial nucleus; 4thv, fourth ventricle; v, vermis

Scale bar, 100 μm in A; 50 μm in B; 1000 μm in C; 500 μm in D, E

In chapter four, we used *Pap* mutant mouse cerebellum, in which the expression of α-synuclein (SNCA) is absent, to explore the fate of $SNCA^+$ cells in cerebellar primordium. IHC in the adult control cerebellum shows SNCA expression in the granular layer of the axonal terminal, while SNCA is not expressed in the *Pap*-null mouse. The lack of SNCA expression in *Pap*-null cerebellum has also been confirmed by Western Blot. Thus, *Pap*-null mouse embryo is a promising model to understand the role of SNCA in this subset of CN neurons in the NTZ during early cerebellar development. Double immunolabeling with SNCA and p75ntr at E12 showed that the $p75ntr^+$ cells are present in NTZ, but there was no SNCA expression compared with wild type. *Pap*-null mouse behavior and motor activity was monitored for about 15 months, and no obvious motor disability was observed. However, *Pap*-null mice seemed to be more active compared with the wild type mice even at older ages (see chapter four).

To further our understanding of the SNCA expression in early cerebellar development, primary dissociated cerebellar culture at E12, DIV 5 was double immunolabeled with β-tubulin. We found that, in addition to localization in cytoplasm (Fig. 3.10G and 3.14 E), SNCA can also be localized in the nucleus (Fig. 3.14 A–D). Embryonic cerebellar culture in the E10 mouse embryo revealed that SNCA expression is localized in both the cytoplasm (arrow) and nucleus (arrow head) of the cell body and process normally at DIV 6 (Fig. 3.14 E–G); however, at DIV 13, SNCA seems to be aggregated in cells or cell clusters (Fig. 3.14 H–J). SNCA aggregation is a major cause of neurodegeneration (Nah et al., 2015), and therefore it may indicate that there is a spatial and temporal expression of SNCA from the NTZ to the neuronal axon terminals. As indicated before our follow up of the expression of Otx2 in E12-15 cerebellar primordium was confirmed the presence of these cells (Fig. 3.5 and 3.6). The presence of Otx2 positive cells in cerebellar primordium at E18 also was seen (data not shown). All these show the presence of the aforementioned cells in cerebellar primordium beside down regulation of SNCA in NTZ.

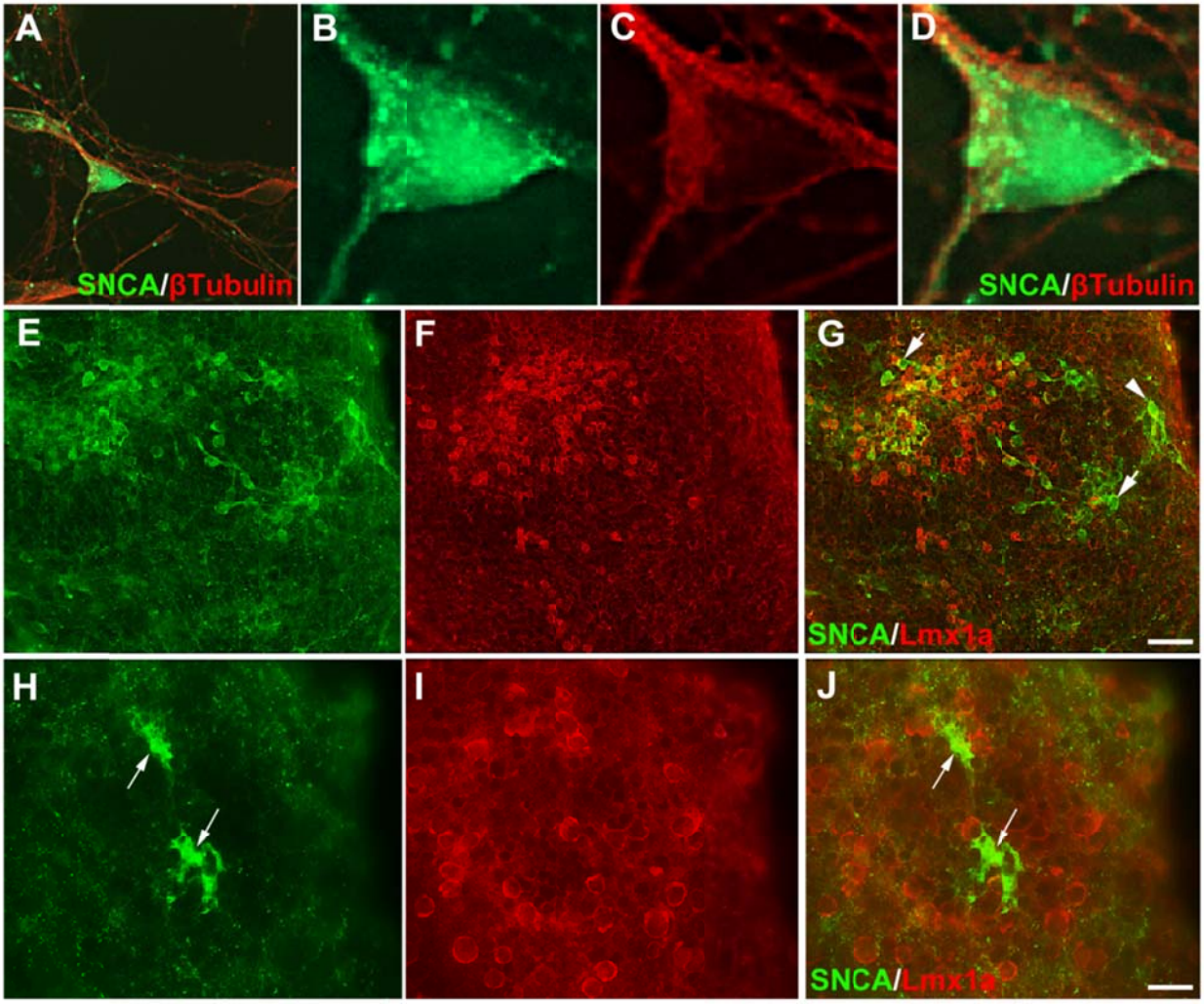

Figure 3. 14 Double immunofluorescence labeled with SNCA (green) and Lmx1a (red) shows aggregation of SNCA in embryonic and cerebellar culture

A–D. Primary dissociated cerebellar culture of E10 mice embryo after 5 days *in vitro* (DIV 5), double immunofluorescence-labeled with SNCA and βtubulin, shows SNCA localization in cytoplasm and nucleus

E–G. Primary dissociated cerebellar culture of E10, after 6 days *in vitro* (DIV 6), double immunofluorescence-labeled for SNCA (green; E) and Lmx1a (red; F), and merged in G. The normal appearance of $SNCA^{+}$ cells form a distinct population of CN neurons from the rhombic lip-derived cells ($Lmx1a^{+}$) and are not overlapped.

H–J. Primary dissociated cerebellar culture of E10, after 13 days *in vitro* (DIV 13), double immunofluorescence-labeled for SNCA (green; H) and Lmx1a (red; I), and merged in (J) shows aggregated form of SNCA in cell or cluster.

Scale bar, 50 μm in E–G; 20 μm in H–J

According to our first understanding of spatial and temporal expression of SNCA, and explore whether $SNCA^+$ cells undergo programmed cell death, cleaved caspase-3 IHC was performed and the results showed that some $SNCA^+$ cells in the NTZ express cleaved caspase-3 (Fig. 3.15 A-D). Western blot analysis revealed activation of caspase 3 in embryonic cerebellum (Fig. 3.15 A). In early embryonic cerebellar sections, cleaved caspase-3 was present in a combination of neurons and neuronal fibers and direct toward the subpial surfaces at the mid-point between the rostral and caudal cerebellar primordium (Figs. 3.12 E, and 3.15 A–D). Cleaved caspase-3 activity is also high in the ophthalmic component of the trigeminal ganglion, a source of early afferent to cerebellar primordium (Fig. 3.15 F–G). Trigeminal ganglion is outlined in whole mount immunohistochemistry with NAA (Fig. 3.15 E). However, the expression of Otx2 in E12-15 cerebellar primordium was confirmed the presence of these cells (Fig. 3.5 and 3.6) and suggests another role for cleaved caspase-3 beside programmed cell death marker.

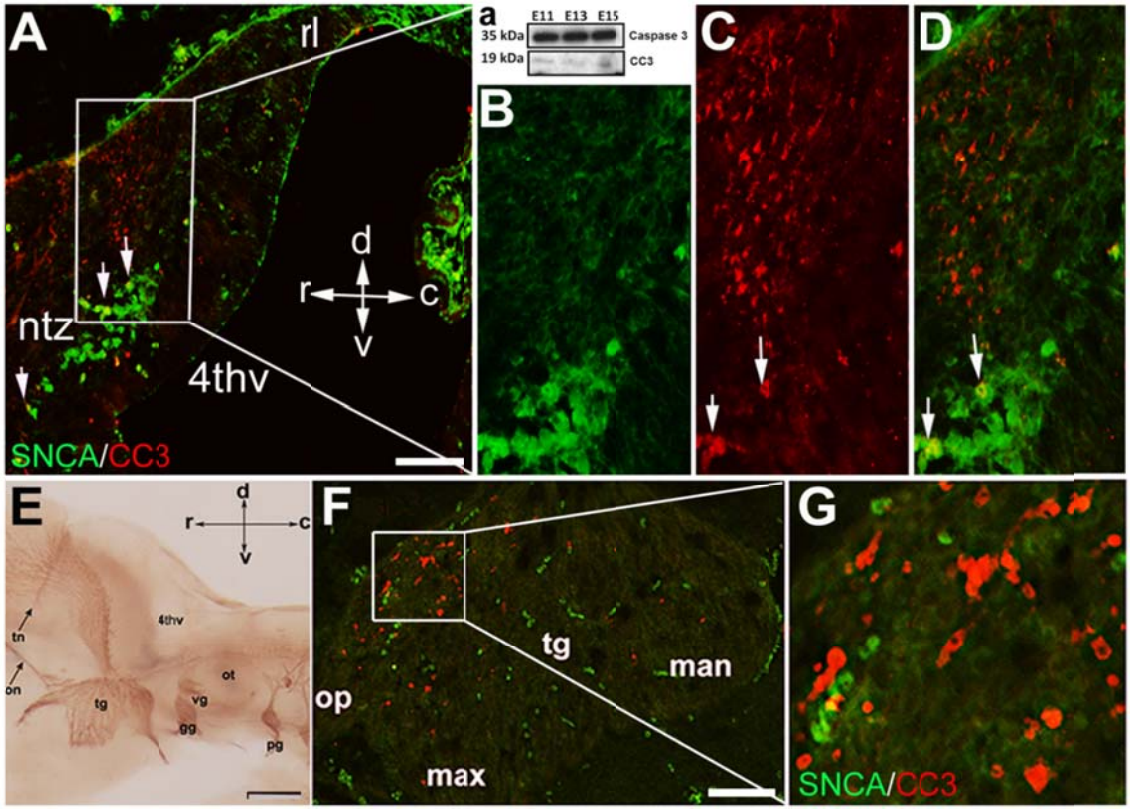

Figure 3. 15 Double immunolabeling for SNCA (green) and cleaved caspase-3 (CC3, red) in the sagittal section of E12 embryo

A-D. Double immunolabeling for SNCA (green) and cleaved caspase-3 (CC3, red) in the sagittal section of the E12 embryo shows activation of caspase-3. This indicates dying cells during early cerebellar development. The boxed region in **A** is shown at higher magnification in **B** (SNCA) and **C** (CC3) and merged in **D**, shows activation of caspase-3 in neurons and fiber combinations in the core of the cerebellar primordium

a. Immunoblots of total caspase 3 and cleaved caspase 3 from cerebella lysate of the embryos at different embryonic days, E11, E13 and E15 indicate caspase 3 activation during early cerebellar development

E-G. The lateral aspect of the mouse embryo at E10, whole-mount IHC with NAA 3A10 showing outline of trigeminal ganglion. Double immunolabeling for SNCA (green) and cleaved

caspase-3 (cc3, red) at sagittal section of E12 embryo trigeminal ganglion shows activation of CC3 is almost localized in the ophthalmic division (op) of the trigeminal ganglion. The boxed region in F is shown at higher magnification in **(G)**.

Abbreviations: gg, geniculate ganglion; ntz, nuclear transitory zone; max, Maxillary division of trigeminal ganglion; man, mandibular division of trigeminal ganglion; ot, otocyst; rl, rhombic lip; vg, vestibular ganglion; tg, trigeminal ganglion; 4thV, fourth ventricle; r, rostral; c, caudal; d, dorsal; v, ventral

Scale bar: 50 μm in A,F; 200 μm in E; 20 μm B–D, G

3.4 Discussion

In this study, we investigated the mesencephalic-derived CN neurons during early cerebellar development. We showed that a novel subset of CN neurons ($SNCA^{+}/Otx2^{+}$ neurons) run from the dorsal mesencephalon and cross the isthmus to the NTZ as early as E9. This suggests the presence of a new germinal zone during cerebellar neurogenesis. Further, these cells are $Otx2^{+}/p75ntr^{+}/Wnt1^{+}/TrkA^{+}$, suggesting that this subset of CN neurons is possibly derived from the mesencephalic neural crest population. This novel subset of CN neurons are accompanied by the nerve fibers that express activated caspase-3, restricted within the cerebellar primordium core. Our results indicate that this subset of the CN neurons/fibers is present until E15 at the cerebellar primordium core while expressing Otx2 and SNCA down regulated. The fibers terminate at the midway point of the rostrocaudal subpial surface. This suggests that the mesencephalic derived $SNCA^{+}/Otx2^{+}/p75ntr^{+}$ combination of neurons/fibers possibly plays a role as signaling center during cerebellar development.

Early subset of CN neurons originate from the mesencephalon

Recent genetic fate mapping studies suggested that most CN projection neurons arise from the rhombic lip (Ben-Arie et al., 1997b, Machold and Fishell, 2005, Fink et al., 2006a). The transcription factor expression patterns suggest that CN neurons migrate from the rhombic lip to the NTZ through a subpial stream pathway while sequentially expressing the genes *Pax6*, *Tbr2,* and *Tbr1* (Marzban et al., 2015, Fink et al., 2006a).

The origin of the $SNCA^+$ neurons is currently not clear, but the rostral continuity of these neurons from the NTZ indicates that they may arise from the mesencephalon. $Tbr1^+/Lmx1a^+$ neurons are born in the rhombic lip at E9, but they do not reach the NTZ until around E10 (Manto et al., 2012, Fink et al., 2006a). Our study showed that $SNCA^+$ cells are a group of differentiating neurons (NAA^+) present in NTZ at E9, before the arrival of any neurons that originate in the rhombic lip. Although, most $SNCA^+$ neurons are not $Lmx1a^+$, some of the neurons are co-labeled with SNCA. This suggests that in the early stages of CN neurogenesis, the pattern of protein expression in $SNCA^+$ neurons is changing, possibly with SNCA being down-regulated and $Lmx1a^+$ being up-regulated, or this may not occur at all (Fig. 3.2). The NTZ is an intercalated area of the $SNCA^+/Lmx1a^+$ cells, and it shows the continuation of $SNCA^+$ to the rostral end and $Lmx1a^+$ to the caudal end as a source of origin.

To further study this hypothesis, we investigated *Otx2* expression in this subset of neurons in the developing cerebellum. *Otx2* has been shown to be involved in prosencephalon and mesencephalon establishment, but not the rhombencephlon (Simeone et al., 1992, Alvarado-Mallart, 2005). It is shown that *Otx2* is required for the development of the forebrain and midbrain, while *Gbx2* is necessary for development of the anterior hindbrain (Li and Joyner, 2001, Joyner et al., 2000b). Our results indicate that the $SNCA^+$ cells in the NTZ express *Otx2.*

In addition, *Wnt1* which is express in mesencephalon (Li et al., 2005) is co-expressed with *Otx2*$^+$ cells in rostral cerebellar primordium, but not *Fgf8*. Considering that mesencephalic cells are *Otx2*$^+$ (Kimura et al., 1997), we suggest that SNCA$^+$ and Otx2$^+$ cells in cerebellar primordium originate from the mesencephalon. Using embryonic culture with inserted DiI, we clearly showed that mesencephalic derived cells through the rostral end migrate to the cerebellar primordium. This strongly suggests that the caudal mesencephalon may play a role as a third germinal zone, which forms a subpopulation of CN neurons that likely predates all neurogenesis in the cerebellar primordium. These finding are in corroboration with the findings of Nichols and Bruce (Nichols and Bruce, 2006b) which suggest the lineage of Wnt-1 lip give rise to neural crest and hind brain migration.

SNCA$^+$/Otx2$^+$ cells with p75ntr$^+$/NAA$^+$ cell and fibers reveal a combination of neurons and neuronal fibers at the core of cerebellar primordium

The neuronal precursor in the mesencephalon arises from neuroepithelium and neural crest cell population (Narayanan and Narayanan, 1978b, Jacobson, 2013, Stainier and Gilbert, 1989). In the mesencephalon, the mesencephalic nucleus of the trigeminal nerve is the large sensory neurons, which are derived from the neural crest (Narayanan and Narayanan, 1978b, Lumsden et al., 1991, Baker et al., 1997). They are the earliest neuronal outgrowths that have been detected in the central nervous system, and are the first differentiated neurons in the mouse brain at E9 (Narayanan and Narayanan, 1978b, Stainier and Gilbert, 1989). It is also suggested that *Otx2* is expressed in migrating mesencephalic neural crest cells (Kimura et al., 1997). We showed that SNCA$^+$/Otx2$^+$ cells are *Wnt1*$^+$ that is express in precursors of the neural crest and substantially contribute to the CN (Chai et al., 2000). In addition, we showed that SNCA$^+$/Otx2$^+$

cells are p75ntr^{+} and TrkA^{+}, both are the nerve growth factor (NGF) receptor expressed in neural crest cells (Tomellini et al., 2014, Harris and Erickson, 2007, Wehrman et al., 2007, Schwarz et al., 2008). Therefore, this suggests that SNCA^{+}/Otx2^{+}/p75ntr^{+}/TrkA^{+} neurons in the NTZ arise from the mesencephalic nucleus of the trigeminal nerve. The neural crest cells are a highly migratory multipotent cell population that arises from the neural fold and gives rise to a wide variety of cell types (e.g. (Bronner and LeDouarin, 2012, Sauka-Spengler et al., 2007, Mayor and Theveneau, 2013)). A neural crest-derived subpopulation in the CNS represents a great research potential because neural crest-derived cells may have the capacity for neurogenesis and regeneration, e.g. in the olfactory system (Forni and Wray, 2012, Katoh et al., 2011).

Transient expression of SNCA in mesencephalic-derived CN neurons

During cerebellar development, the prominent SNCA expression in CN neuron somata is transiently initiated, and at around E15–16, its expression in the developing cerebellum may diffuse into neuronal fibers (Fig. 3.13). This dynamic expression pattern is in agreement with other studies on SNCA expression patterns in embryos (Zhong et al., 2010b). In our study, we did not detect SNCA expression in any neuronal somata in the cerebellar cortex and CN during perinatal development. It has been suggested that the SNCA may be involved in neuronal migration, neural cell fate and differentiation (Schneider et al., 2007). However, our study on the PAP/SNCA-null mouse (see in chapter three) showed that the mesencephalic-derived cells migrate to the NTZ, which indicates that a lack of SNCA is not involved in the migration. Therefore, the cytoarchitecture and function of the cerebellum was not affected despite the absence of SNCA, and there may be a redundancy using other molecules to compensate for the loss of SNCA function.

The nuclear localization of SNCA was also reported in several studies in both physiological and pathological situations (Specht et al., 2005, Kontopoulos et al., 2006). SNCA overexpression and its aggregated form have been shown to be involved in neurotoxicity (Azeredo da Silveira et al., 2009, Zhong et al., 2010b, Kontopoulos et al., 2006). Our results demonstrate SNCA aggregation in the embryonic cerebellar culture of an E10 cerebellar primordium after 13 days *in vitro* (DIV 13), but not at DIV 6. The $SNCA^{+}/Otx2^{+}/p75ntr^{+}$ combination of neurons/fibers at the core of the cerebellar primordium showed an activated CC3 that began from the mesencephalon and terminated at the midpoint of the rostrocaudal cerebellar primordium.

The transient presence of $SNCA^{+}$ cells in the core of the early cerebellar primordium suggests that they have a temporary regulatory role between the rostrodorsal and caudoventral domains in the developing cerebellum. The neuronal fibers probably originate from early CN neurons or from pioneer axons that originate from the trigeminal nerve with or without synapses containing $SNCA^{+}/Otx2^{+}$ cells that terminate on the subpial surface at the point that rhombic lip-derived $Lmx1a^{+}$ CN changes direction from tangential migration to approximately radial migration. Therefore, it may provide a substrate pathway that directs the $Lmx1^{+}$ cells to migrate to their destination in the NTZ. Given that rhombic lip-derived $Lmx1a^{+}$ neuron migration to the NTZ is paved by pioneer neural fibers, our first expectation was that they may disappear soon after migration because of the expression of CC3 and these $CC3^{+}$ fibers may play a part in axonal guidance and neuronal migration during early cerebellar development. To our surprise, when we follow the expression of Otx2 in these cells from E12 to E15, the presence of Otx2 positive cells persists while SNCA expression is down regulated and questioned the activation of CC3 as program cell death marker and highlighted the survival role and axonal guidance of CC3

during development (Fig. 2.5, 2.6). In addition, this set of pioneer neural fibers persists and can be traced up to the perinatal period of cerebellar development. Thus, this set of pioneer neural fibers may have dual functions, and its second role probably points to the midway position of subpial termination and indicates the putative lobule VI/VII at the paramedian sulcus. The significance of this termination around the perinatal period at the paramedian sulcus probably causes an area with no cerebellar cortex, known as an acortical area, in some adult cerebelli, such as in the bat (Kim et al., 2009a) and the naked mole rat (Marzban et al., 2011). However, this does not happen for all species (e.g. mouse (Marzban et al., 2008a)). It is not clear whether the presence of this set of fibers until around the perinatal period is involved in acortical area formation in paramedial sulcus in the cerebellum. Finally, the prominent presence of early combinations of neurons/fibers may indicate an organizing center at the cerebellar primordium core, and continuation neuronal fibers could be a landmark for anterior and posterior development.

3.5 Conclusion

Our study suggests that a subset of CN neurons that is $SNCA^{+}/Otx2^{+}/p75ntr^{+}$ originates from the mesencephalic neural crest, which is a possible new germinal zone for cerebellar development. We showed that this subset of the CN neurons accompanies nerve fibers that are present at the core of cerebellar primordium and terminates at the midway of the rostrocaudal subpial surface. This suggests that they play a role as a secondary organizer that may act as a signaling center. This center probably directs rhombic-derived cells to the NTZ and also organizes the cerebellum into anterior and posterior divisions at the level of lobule VI/VII. We are only beginning to understand the role of mesencephalic-derived CN neurons that accompany

the neuronal fibers at the core of the cerebellar primordium, and further studies are required to determine the precise molecular mechanism and functional significance of this putative signaling center.

CHAPTER FOUR: CEREBELLAR CORTEX PATTERNING IN PROSTATIC ACID PHOSPHATASE MUTANT MOUSE WITH ABSENCE OF THE α-SYNUCLEIN GENE

This Manuscript is submitted to Plos One (the submitted version includes data produced in another laboratory). PLOS One is a peer-reviewed open access scientific journal published by the Public Library of Science

4.1 Introduction

α-synuclein (SNCA protein, the *Snca* gene encodes α -synuclein, a small 140 amino acid protein that can be both lipid associated and free in the cytoplasm (Farrer, 2006)) is one of several major members of intracellular fibrillary proteins, abundant protein in presynaptic axon terminals and important for brain normal function (Maroteaux et al., 1988). Synuclein family, unfolded proteins that can transiently bind to lipid membranes and acquire a partial α-helical conformation, are comprised of α-, β-, and ɣ-synuclein, and synoretin (Tofaris and Spillantini, 2007). It was found first in Torpedo californica's acetylcholine vesicles and suggested to have a role in dopaminergic neurotransmission and synaptic plasticity (Maroteaux et al., 1988, Clayton and George, 1999). When SNCA aggregates in the brain (forming oligomers and insoluble fibrils with increased ß-sheet configuration, Lewy bodies and non-Amyloid β component) it can result in a subset of neurodegenerative disorders like Parkinson's disease (PD), dementia with Lewy bodies (DLB), Alzheimer's disease (AD) and other synucleinopathies (Iwai et al., 1995, Iwatsubo, 2003).

Many of the proteins hypothesized to be important in the progression of neurodegeneration play direct or indirect roles in neurodevelopment (Rogers and Schor, 2010b). In a study conducted on the developing human from fetus to adulthood, it is concluded that the expression of α-synuclein (SNCA) observed and condensed first in neuronal cell bodies of cortical plate at 11 weeks, then in the hippocampus, basal ganglia, and brain stem at 20 weeks and persist for few years after birth (Raghavan et al., 2004). In the cerebellum, it expresses in granular layer and molecular layer which starts at 21 weeks and continues until adulthood (Raghavan et al., 2004). In the study was done by Raghavan et al., it is demonstrated the expression of SNCA in brain (anatomical and subcellular localization) varies with age, and starts

disappearing from the neuronal cytosol in early fetus, and only presents in neuronal processes in older fetuses and adults while its role changes from stem cell fate and differentiation to synapse plasticity, synaptogenesis, and neurotransmission (Raghavan et al., 2004).

In mice, the expression of SNCA in developing brain has been detected as early as E 9.5 in the marginal zone of the neocortex and later in the subplate (Zhong et al., 2010a). In the cerebellum, the expression of SNCA is condensed initially in the cerebellar nuclei and then condensed in the Purkinje cells. The reason for this spatio-temporal expression pattern underscored by the authors as a response to the neuronal migratory pathways and the formation of the synapse connections (Zhong et al., 2010a). In the mice lacking SNCA, the regulatory role of SNCA is shown through depletion of the presynaptic vesicular pool (Cabin et al., 2002).

Thiamine monophosphatase (TMPase, also known as fluoride-resistant acid phosphatase) is a classic histochemical marker of small-diameter dorsal root ganglia neurons (Zylka et al., 2008). It has been reported that TMPase is identical to the transmembrane isoform of prostatic acid phosphatase (PAP), an enzyme with unknown molecular and physiological functions (Zylka et al., 2008). As an important marker for prostatic carcinoma, PAP was identified long before the introduction of prostate specific antigen (PSA) (Whitesel et al., 1984, Veeramani et al., 2005, Araujo and Vihko, 2013). It is indicated that mice deficient in transmembrane PAP display increased GABAergic neurotransmission beside increase in striatal dopamine synthesis and neurological alterations (Nousiainen et al., 2014). Neurodegenerative disorders with synucleinopathies are accompanied with dopaminergic neuron loss (Bianco et al., 2002). Interestingly, PAP has stronger antinociceptive effects than the opioid analgesic morphine and has been suggested to use for the treatment of chronic pain (Zylka et al., 2008).

In this study to question any neurological abnormalities related to cerebellum, we investigated the cerebellar cortex patterning to indicate any changes in form of stripes and patterns compartmentation in PAP mutant mice, to uncover the possible role of PAP and SNCA in the cytoarchitecture and function of the cerebellum.

4.2 Material and Methods

Animal maintenance

All animal procedures were performed in accordance with institutional regulations and the *Guide to the Care and Use of Experimental Animals* from the Canadian Council for Animal Care. *PAP* KO mice were obtained from Dr. Pirkko Vihko, University of Helsinki, Finland. All control wide types CD1 mice were obtained from Central Animal Care Service, University of Manitoba. Animals were kept at optimum temperature and relative humidity (18-20˚C, 50-60%) on a light and dark cycle (12:12 h) with free access to food and water. The midday of the vaginal plug discovery was designated embryonic day 0.5 (E0.5) and the day of birth postnatal day 0 (P0). Pregnant females were anesthetized with 40% isoflurane, UPS Baxter Co. Mississauga, Ontario, Canada) and killed via cervical dislocation. The embryos at E 12.5 (n=6, $Pap^{-/-}$, 3 $Pap^{+/+}$) were carefully dissected, placed immediately in ice-cold phosphate buffered saline (PBS) to remove blood, and then fixated overnight in the fixation solution (4% paraformaldehyde (PFA)). For postnatal brain sample collection, mice at P60 (n=20 10 $Pap^{-/-}$, 10 $Pap^{+/+}$) were transcardially perfused at first with ice-cold PBS and followed by the 4% PFA. Then brains removed from skull and placed in the same fixation solution for overnight.

Sections immunohistochemistry and immunofluorescence

The embryos and postnatal brains were transferred to the gradient 10%-20%-30% sucrose until they sank at the bottom of the container. Then they were embedded in clear frozen section compound (OCT: VWR, USA), were frozen at -80 ˚C and cut at 20 µm via cryostat microtome. The sections were placed on slides covered with a coating solution (0.05% chromic potassium sulfate and 0.5% gelatin) or floated in sterile PBS to be utilized for immunochemistry (IHC) process as explained in our previous studies (Bailey et al., 2014a, Bailey et al., 2013b). Antibody dilutions were used as follows: SNCA (sc-69977, Santa Cruz) 1:500 (Zhong et al., 2010a), p75NTR (8238, Cell Signaling) 1:1000 (Rahimi Balaei et al., 2016a). Two anti-calbindin (Calb1) antibodies were used (in the cerebellum, Calb1 is entirely expressed in Purkinje cells): Rabbit polyclonal anti-calbindin D-28K antiserum (anti-Calb1, diluted 1:1,000, Swant Inc., Bellinzona, Switzerland), and mouse monoclonal anti-calbindin (anti-Calb1, diluted 1:1,000, Swant Inc., Bellinzona, Switzerland) (Bailey et al., 2013b, Bailey et al., 2014a). Anti-zebrin II (a gift from Dr. Richard Hawkes, University of Calgary, Calgary, AB, Canada) is a mouse monoclonal antibody that was produced by immunization with a crude cerebellar homogenate from the weakly electric fish Apteronotus. We used it directly from spent hybridoma culture medium (diluted 1:200) (Bailey et al., 2013b, Bailey et al., 2014a, Brochu et al., 1990). Fluorescent detection was performed using followed antibodies: Alexa Fluor® 568 Goat Anti-Rabbit IgG (H+L), Alexa Fluor® 488 Chicken Anti Mouse IgG (H+L) (A-11036, A21200, Life Technologies) 1:1000. Detection of peroxidase IHC was also performed as described previously using HRP conjugated goat anti rabbit IgG and goat anti-mouse IgG (H+L) antibodies (EMD Millipore Corporation, 12-348 and AP308P, respectively) 1:500, and developed with DAB (3,3'-diaminobenzidine) solution (Sigma, St. Louis MO, USA).

Western blotting analyses

Equal amount of proteins (n=6, *Pap*$^{-/-}$, 3 *Pap*$^{+/+}$) were separated by SDS/PAGE in 10–15% precast gels (Bio-Rad, Hercules, CA, USA) and transferred onto the PVDF-membrane. For the Western blot analysis, membranes were blocked in 5% nonfat dry milk (NFDM) in TBS containing 0.02% Tween 20 (TBST) and then incubated overnight at 4°C with primary antibodies as follows: α-synuclein (sc-69977, Santa Cruz) 1:2000. Secondary antibodies as follows: HRP conjugated goat anti-mouse IgG (AP308P, Millipore) 1:6000. Binding was assessed using DAB (3,3'-diaminobenzidine) solution (Sigma, St. Louis MO, USA).

PCR analysis

To study the expression of SNCA in *Pap*$^{-/-}$and *Pap*$^{+/+}$, total DNA from cerebellum (n=6, *Pap*$^{-/-}$, 3 *Pap*$^{+/+}$) was extracted using a kit (AccuStart^{TM} II Mouse Genotyping Kit, Cat# 95135-500, Quanta BioSciences, Inc. MD, USA), according to manufacturer's instructions. DNA quality and quantity were determined by measuring the absorbance at 260 and 280 nm using NanoDrop ND-1000 UV- 19 Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples had an absorption ratio A260/A280 between 1.8 and 2.2. DNA (1 μg) from each sample was used.

To amplify the SNCA gene, PCR reactions were performed in a T3000 thermocycler (Biometra, Göttingen, Germany) using AccuStart^{TM} II GelTrack^{TM} PCR SuperMIX (2X) (Cat# 95136-500, Quanta BioSciences, Inc. MD, USA) in a final volume of 25 μL. There were three sets of primers to amplify across an intron to probe genomic DNA for SNCA. Then PCR products were run on PCR agar gel and detected for the target gene (SNCA) bands to distinguish differences in PAP-WT from PAP-KO mice. [To confirm the simultaneous deletion of *Snca* with

Pap, the expression of *Snca* in *Pap* $Pap^{-/-}$ and $Pap^{+/+}$ was studied. PCR analysis was done in Dr. Fiona Parkinson lab and data are not shown in the result part].

Imaging and figure preparation

For bright field microscopy, images were captured using Zeiss Axio Imager M2 microscope (Zeiss, Toronto, ON, Canada). Images were then analyzed with a Zeiss Microscope Software (Zen Image Analyses software) (Zeiss, Toronto, ON, Canada). For fluorescence microscopy of the embryonic entire cerebellum sections, a Z2 Imager Zeiss Fluorescence microscope (Zeiss, Toronto, ON, Canada) equipped with a camera was used to capture the images. Images were then analyzed using Zen software. Images were cropped, corrected for brightness and contrast, and assembled into montages using Adobe Photoshop CS5 Version 12.

4.3 Results

SNCA expression in cerebellum of $Pap^{-/-}$ mouse and wide type controls (Zone and Stripe Pattern)

The expression of SNCA in different part of central nervous system (cerebrum, cerebellum, brain stem and spinal cord) was evaluated at protein level by Western blotting. Western blot analysis at P=60 showed SNCA expression in all different parts (cerebrum , cerebellum, brain stem and spinal cord) of the control $Pap^{+/+}$ and none of the $Pap^{-/-}$ (Fig. 4.1 A). The expression of SNCA was also evaluated by immunohistochemistry of transverse cerebellar sections at P= 60 and confirmed Western blot results. In the cerebellum of control $Pap^{+/+}$, SNCA was expressed in the axon terminals of the mossy fibers in the granular layer (Fig. 4.1 B).

Transverse section through the anterior cerebellum immunoperoxidase-stained for SNCA showed strong immunoreactivity in the granular layer in an array of parasagittal stripes (lobules III and V; Fig. 4.1 C). The *Pap*$^{-/-}$ transverse cerebellar section was immunolabeled with SNCA and showed no immunoreactivity in granular layer (Fig. 4.1 D) or in anterior zone (Fig. 4.1 E).

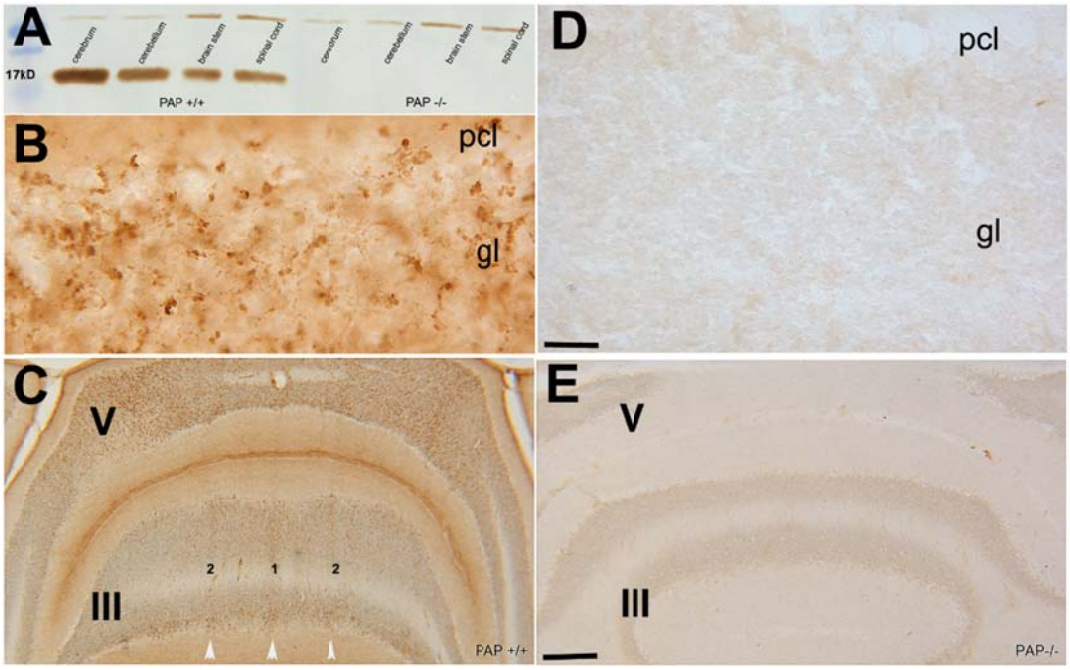

Figure 4. 1 SNCA expression in cerebellar vermis of adult WT and Pap null mice.

A. Immunoblotting showed lack of the SNCA expression in *Pap* null cerebellum, cerebrum, brainstem, and spinal cord tissue at P= 60 in comparison to the control.

B-C. SNCA immunoperoxidase staining of a transverse section through the anterior cerebellum at P= 60 shows strong immunoreactivity in the granular layer in the terminals of mossy fibers afferents (B) in an array of parasagittal stripes in anterior zone (C).

D-E. The *Pap* null cerebellum shows a lack of SNCA expression at low (D) and in higher magnification (E).

Abbreviations: pcl: Purkinje cell layer, gl: granular layer, III and V: lobule III and lobule V

Scale bar: 20 µm in B and D; 200 µm in C and E

The expression of SNCA in embryonic stage in Pap$^{-/-}$ mouse and wide type controls

To determine the expression of SNCA during early development, double immunofluorescence staining performed in sagittal cerebellar sections at E12 with SNCA and P75NTR (positive immunoreactivity for nuclear transitory zone (NTZ) at early cerebellar development). In control *Pap*$^{+/+}$ (Fig. 4.2 A-C), SNCA immunopositive cells in NTZ (Fig. 4.2 A) and P75NTR immunopositive cells in NTZ (Fig. 4.2 B) were observed and co-labeled (merge one, Fig. 4.2 C). In comparison, the *Pap*$^{-/-}$ sagittal cerebellar sections at E12 showed no immunoreactivity for SNCA (Fig. 4.2 D) while P75NTR immunopositive cells were seen in NTZ (Fig. 4.2 F). No co-labeling of NTZ was seen (Fig. 4.2 G).

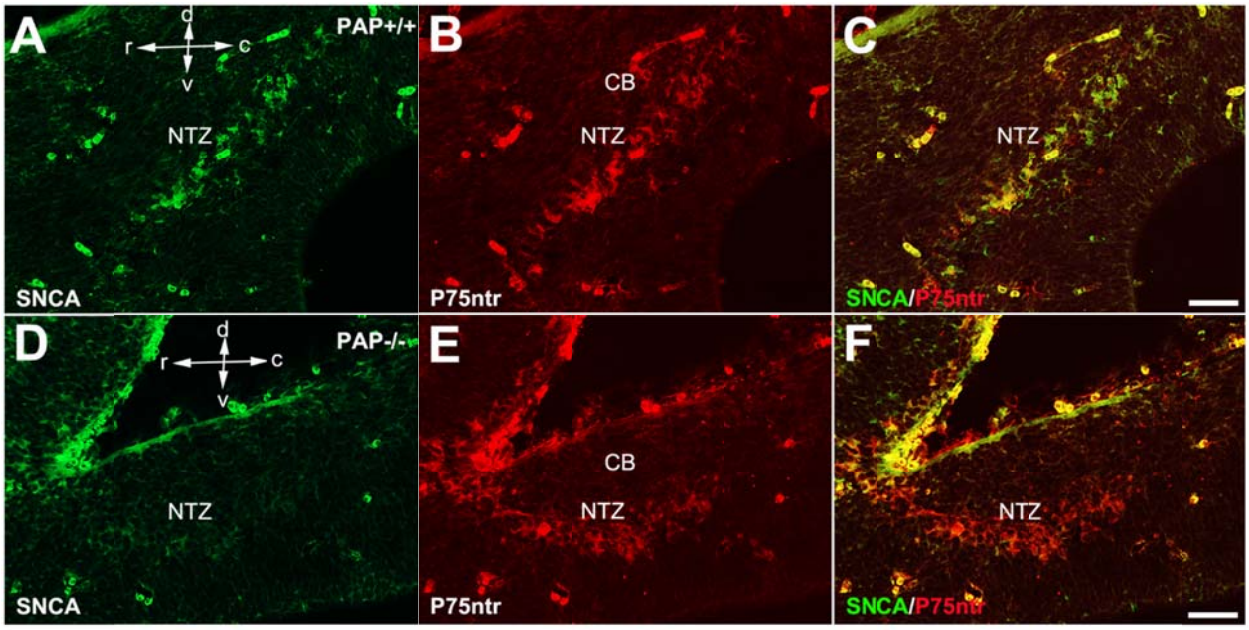

Figure 4. 2 Lack of Snca expression in the Pap mouse at E12

A-F. Double immunostaining with SNCA (green) and P75NTR (red) at E12 in WT (A–C) and *Pap*-null mice (D–F) shows a lack of SNCA expression in NTZ cells (D), while P75NTR cells are present in NTZ cells and confirm the presence of these cells (E).

Abbreviations: CB: cerebellum, NTZ: nuclear transitory zone

Scale bar= 50 μm

The expression of Calb1 and zebrin II in cerebellum of $Pap^{-/-}$; $Snca^{-/-}$ mouse and wide type controls (Zone and Stripe Pattern)

The expression of Calb1 (a marker expressed by all of the Purkinje cells (Bailey et al., 2014a)) is a critical determinant of the precision of motor coordination (Barski et al., 2003) was also evaluated by immunohistochemistry of transverse cerebellar sections at P= 60 and showed no differences between $Pap^{+/+}$; $Snca^{+/+}$ (Fig. 4.3 A) and $Pap^{-/-}$; $Snca^{-/-}$ (Fig. 4.3 B) and appeared all Purkinje cells are present with normal phenotype, arranged Purkinje cell bodies in line and their dendrites were arborized to molecular layer. Immunolabeling of transverse cerebellar sections with zebrin II showed the pattern expression of zebrin II with parasagittal stripes in $Pap^{+/+}$;$Snca^{+/+}$ (Fig. 4.3 C) is the same in anterior (Fig. 4.3 D), central (Fig. 4.3 E), posterior (Fig. 4.3 F) and nodular (Fig. 4.3 G) zones in $Pap^{-/-}$;$Snca^{-/-}$ mice and is normal. The mouse cerebellar cortex is subdivided into four transverse zones—the anterior zone (AZ: ~lobules I–V), the central zone (CZ: ~lobules VI–VII, with two sub-zones—see (Marzban et al., 2008b)), the posterior zone (PZ: ~lobules VIII + dorsal IX), and the nodular zone (NZ: ~ventral lobule IX + lobule X) (e.g., (Sillitoe and Hawkes, 2002) and (Marzban and Hawkes, 2011)).

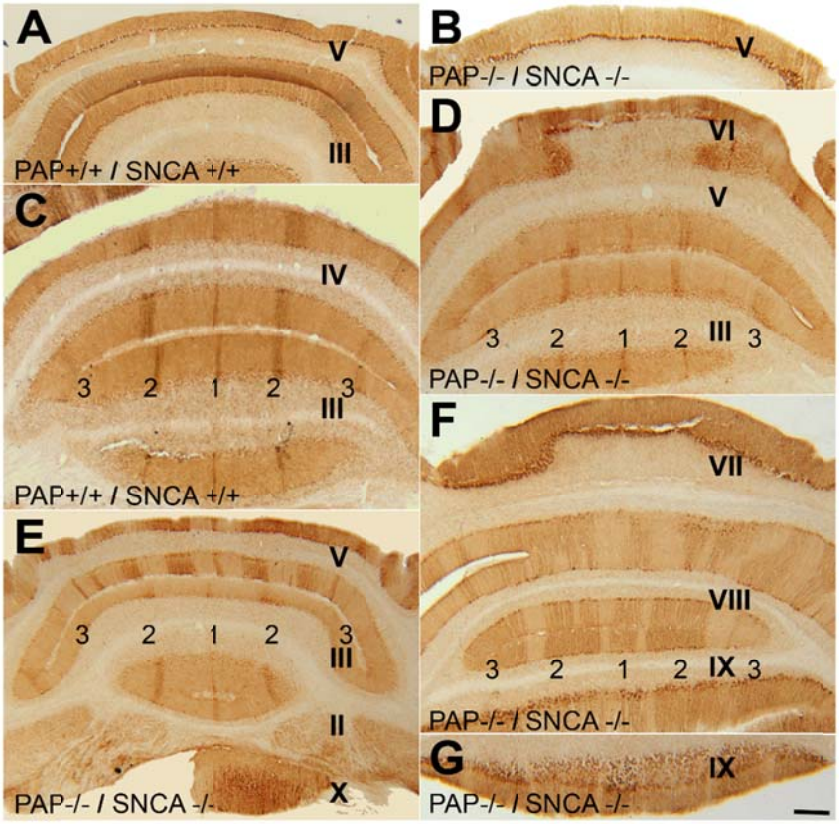

Figure 4. 3 Transverse sections through the adult cerebellum of WT and Pap null mice immunostained with Calb1 and zebrin II.

A-B. Immunohistochemistry with Calb1 (a pan Purkinje cell marker in the cerebellum) shows there is no phenotypic alteration between the WT (A) and *Pap* mutant (B).

C-G. Transverse sections through the cerebellum of the adult control (C) and *Pap* null (D-G) immunoperoxidase stained for zebrin II. The pattern of parasagittal stripes in the anterior zone central zone, posterior zone, and nodular zone are normal. The conventional stripe nomenclature has zebrin II+ stripes called P+, and are numbered from P1+ (indicated by 1) at the midline to P3+ (indicated by 3) laterally(e.g., (Marzban and Hawkes, 2011) and (Kim et al., 2009b)).

Abbreviations:

Scale bar= 200µm

The expression of P75NTR and HSP25 in cerebellum of Pap$^{-/-}$;Snca$^{-/-}$ mouse and wide type controls (Zone and Stripe Pattern)

The expression of P75NTR in central zone (Fig. 4.4 A) and nodular zone (Fig. 4.4 B) in *Pap*$^{+/+}$;*Snca*$^{+/+}$ transverse sections of cerebellum at P= 60 showed immunoreactivity in stripes pattern. In comparison to the *Pap*$^{+/+}$;*Snca*$^{+/+}$, the transverse sections of *Pap*$^{-/-}$;*Snca*$^{-/-}$ cerebellum showed similar pattern of stripes with P75NTR immunopositive Purkinje cells in central (Fig. 4.4 C) and nodular zone (Fig. 4.4 D). The pattern of P75NTR positive immunoreactivity resembles HSP25 expression in same stripes pattern in central and nodular zone of *Pap*$^{-/-}$;*Snca*$^{-/-}$ cerebellum (Fig. 4.4 E,F). HSP25 immunolabeling is absent in the anterior and posterior zones, while expressed in parasagittal stripes in the central and nodular zone of the normal cerebellum (Bailey et al., 2014a).

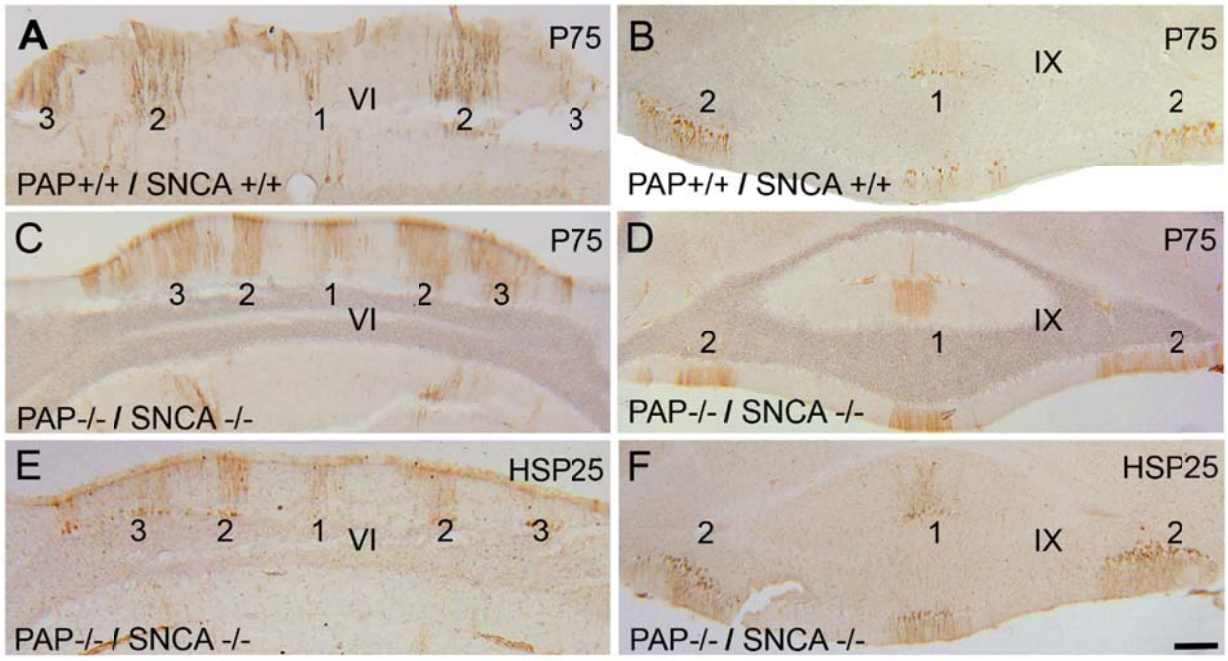

Figure. 4 P75NTR and HSP25 expression in the cerebellum of the WT (A-B) and Pap null mouse (C-F).

A-B. P75NTR immunoperoxidase staining shows pattern of stripe immunoreactivity in central zone (A) and nodular zone (B) in WT cerebellum.

C-D. P75NTR immunoperoxidase staining shows pattern of stripe immunoreactivity in central zone (C) and nodular zone (D) in *Pap* null cerebellum which is comparable with WT.

E-F. Immunoperoxidase staining for HSP25 in transverse sections through the central zone and nodular zone of the *Pap* mutant cerebellum reveals that subsets of Purkinje cells expressing HSP25 are interposed by HSP25 immunonegative Purkinje cells which are comparable with p75NTR expression in WT and *Pap* mutant.

Abbreviations:

Scale bar = 200μm

4.4 Discussion

In this study, we have shown that despite the importance of the expression of PAP and SNCA during development, knock-out mice display normal brain morphology, including stripes and zone patterns of the cerebellar cortex architecture. An initial objective of the project was to identify the expression of SNCA in *PAP* mouse, and the idea of studying the expression of SNCA in *Pap KO* mice was done following our lab interest in cerebellar expression of SNCA during early and postnatal development. One interesting finding is that at different part of the brain (cerebrum, cerebellum, brain stem and spinal cord) there was no expression of SNCA in the *Pap* mouse. SNCA is not expressed in the axon terminals of mossy fibers and confirms the absence of this gene in *Pap* mouse. From our data, it appears that *Pap KO* is also *Snca* knock out. It is hard to call them double knock out since it was not done intentionally. Several reports have shown their findings on the deletion of *Snca*. In studies done on the mutated model of

Snca, it is indicated that although *Snca* is expressed throughout the brain, knock-out mice display normal brain morphology, including number and structure of neurons and under certain conditions, these mice demonstrates Lewy bodies inside of dopaminergic neurons. The defects in dopamine signaling, which may indicate a developmental role for SNCA in the nigrostriatal pathway that is functionally, but not structurally, apparent (Abeliovich et al., 2000, Crosiers et al., 2011). Others indicated the reduction in striatal dopamine and an attenuation of dopamine-dependent locomotor response to amphetamine plus resistance to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is a prodrug to the neurotoxin MPP+, which causes permanent symptoms of Parkinson's disease by destroying dopaminergic neurons in the substantia nigra of the brain) and suggesting that SNCA deficiency results in neuroprotection by reducing stress (Fornai et al., 2005, Dauer et al., 2002, Klivenyi et al., 2006).

As indicated before by Zhong et al. during embryonic stage, SNCA is condensed in NTZ of early developing cerebellum (Zhong et al., 2010a). To examine the possibility of the disappearance of NTZ cells in the current study we found that despite the deletion of this gene, NTZ cells are still there and are labeled by P75NTR while are immunonegative for SNCA. Neuronal proliferation/differentiation is regulated by P75NTR that are mainly expressed during early development (Jiang et al., 2008, Bernabeu and Longo, 2010b, Dechant and Barde, 2002a).

Another interesting finding was by PCR experiment and confirmed the simultaneous deletion of *Snca* with *Pap* while a note of caution is due here since questioning the possibility of deletion of other genes and mainly raises questions about the original strain and / or mutation method.

There is a fundamental cytoarchitecture organization in the cerebellar compartmentation and each gene is expressed, functioned and aligned in the pattern of zones and stripes (Bailey et

al., 2014a, Bailey et al., 2013b, Rahimi Balaei et al., 2016a, Rahimi-Balaei et al., 2018b, Afshar et al., 2017, Sarna et al., 2006b). The best way of studying Purkinje cell degeneration and vulnerability is indicated by the pattern of gene expression in different cerebellar lobules (Afshar et al., 2017, Bailey et al., 2014a, Rahimi Balaei et al., 2016a, Sarna et al., 2003b, Sarna and Hawkes, 2003b, Rahimi-Balaei et al., 2018b). Numerous genes are expressed uniformly or their immunoreactivity are entirely negative in the CZ and NZ (Bailey et al., 2014a, Armstrong et al., 2000b). Here, well-known Purkinje cell markers were used: Calb1, zebrin II, P75NTR and HSP25. The expression pattern of Calb1 showed no differences between normal and mutant mice and was expressed in all of Purkinje cells. The parasagittal striped expression pattern of zebrin II with immunopositive bands from medial to lateral of adult transverse cerebellar sections are termed P1+ to P7+ (Rahimi Balaei et al., 2016a, Marzban et al., 2007) and were identical in different zones. HSP25 is another Purkinje cell marker and it is expressed in the CZ and NZ uniformly at around P12 (Armstrong et al., 2001). By around P15-21 the expression pattern of HSP25 in CZ became striped. It is reported the corticogenesis and development of CZ occurred in slower pace in comparison to the other cerebellar zones (Vastagh et al., 2005). In another study done before, we have shown that P75NTR pattern and protein expression in the adult cerebellar section is comparable with HSP25 expression (Rahimi Balaei et al., 2016a). Surprisingly, the parasagittal stripe pattern expression of P75NTR and HSP25 in the CZ and NZ resembled each other and showed no differences between knockout and WT control cerebellum. All in all were in support of the normal cerebellar compartmentation and morphology in dual *Pap / Snca KO* mice.

Small-diameter neurons are located in the dorsal root ganglia and trigeminal ganglia which are responsible for sensing painful and tissue-damaging stimuli (Woolf and Ma, 2007,

Dubin and Patapoutian, 2010). These neurons express acid phosphatase or TMPase (Knyihár-Csillik et al., 1986, Silverman and Kruger, 1988). Prostatic acid phosphate is used as an indicator for diagnosis and treatment response in prostate carcinoma patients (Veeramani et al., 2005, Watson and Tang, 1980, Dattoli et al., 1999, Araujo and Vihko, 2013). Thiamine-dependent processes are diminished in brains of patients with several neurodegenerative diseases. It is indicated prostatic acid phosphatase is required for the antinociceptive effects of thiamine (Hurt et al., 2012). The decline in thiamine-dependent enzymes (TMPase, transmembrane isoform of PAP (Quintero et al., 2007)) result in the antioxidant reversal and amelioration of other forms of oxidative stress lead to neurodegenerative disease (Gibson and Zhang, 2002). Thiamine-dependent processes are critical in glucose metabolism and reductions in brain glucose metabolism and increased oxidative stress invariably occur in Alzheimer's disease and thiamine (vitamin B1) deficiency. Both conditions cause irreversible cognitive impairment; their behavioral consequences overlap but are not identical and implicate the role of thiamine in oxidative stress, protein processing, peroxisomal function, and gene expression (Gibson and Blass, 2007, Sang et al., 2018). Beside role of PAP in neurodegeneration, it is reported that *Pap* knockout mice have normal acute pain sensitivity but enhanced sensitivity in chronic inflammatory and neuropathic pain models (increased thermal hyperalgesia and mechanical allodynia) (Zylka et al., 2008). In addition, PAP introduced as a neglected ectonucleotidase (extracellular adenosine production by dephosphorylation of the extracellular AMP to adenosine) could regulate diverse physiological processes that are dependent on adenosine (Zimmermann, 2009, Yegutkin et al., 2014). TMPase deficient mice display increased GABAergic transmission and neurological alterations (Nousiainen et al., 2014).

The most interesting aim of this experiment to pursue was the lack of an unrelated protein (SNCA) which is perhaps a consequence of the *Pap* gene targeting (are they located closely in the genome?) in *Pap KO* mice. These findings demonstrate the normal appearance of the stripe and zone patterns of the genes involved in cerebellar cortex architecture and compartmentation in *Pap*$^{-/-}$*;Snca* mice and if there is an unintentional double knockout, it has been raised an important issue for future research; what else is missing?

4.5 Conclusion

To conclude, the most obvious finding to emerge from this study is that *Pap* and *Snca* dual KO mice have normal cerebellar morphology. These findings will be of interest to clinicians who are working on Parkinson's disease, Alzheimer's disease or other synucleinopathies.

CHAPTER FIVE: The Neurotrophin Receptor p75 Expression in the Cerebellum of the Lysosomal Acid Phosphatase 2 Mutant Mouse (*nax* - Naked-Ataxia Mutant Mouse)

This article published in International Journal of Molecular Sciences (Maryam Rahimi Balaei, Xiaodan Jiao, Niloufar Ashtari, Pegah Afsharinezhad, Saeid Ghavami, Hassan Marzban. Cerebellar Expression of the Neurotrophin Receptor p75 in Naked-Ataxia Mutant Mouse. Int J Mol Sci. 2016 Jan 15;17(1). pii: E115. doi: 10.3390/ijms17010115). The International Journal of Molecular Sciences is a peer-reviewed open access scientific journal covering research in chemistry, molecular physics, and molecular biology.

We described that the expression of the p75NTR (protein 75 neurotrophin receptor) in the primary neurons that originated from mesencephalon was seen in nuclear transitory zone (NTZ) as early as E9 and also in the neuronal and nerve fibers at the future putative VI and VII lobules of the cerebellar primordium. The expression of p75NTR continued until E18 in the cerebellar primordium when checked in mice embryos from E9-18. In chapter three we explained about the expression of p75NTR in embryonic stage. To trace the distribution and pattern of p75ntr expression we used postnatal cerebellum in normal and Acp2 mutant mice with cerebellar defect. Acp2 is expressed in mesencephalic derive cerebellar nuclei neurons (data not shown). Acp2 mutant mice which are as a result of spontaneous mutation in "Lysosomal Acid Phosphatase 2" (Acp2) gene. Lysosomal acid phosphatase 2 (Acp2) is a key enzyme in cerebellar development, and it is implicated in a variety of neurological disorders including progressive supranuclear palsy and juvenile neuronal ceroid lipofuscinosis, also known as Batten disease (a disease induced by mutations in the lysosomal protein ceroid-lipofuscinosis neuronal 3 (CLN3)). In one of our lab publications we have shown the pivotal role of the ACP2 expression during cerebellar development when it expresses in the caudal mesencephalon and cerebellar primordium. Acp2 mutant is also called nax mutant mouse which stands for "Naked-Ataxia Mutant Mouse" and this mouse shows severe cerebellar defects including ataxia (lack of voluntary coordination of muscle movements), reduced size and abnormal lobulation as well as Purkinje cell degeneration. Many of the ataxia are with Purkinje cell degeneration. In this chapter we examined the expression of p75NTR in 171 the normal and Acp2 mutant mice. We showed that the expression of p75NTR protein during embryonic stage (chapter three) and it has been indicated that the expression of p75NTR has a role in neuronal survival, development and function. It is also reported that in the pathological conditions the upregulation of p75NTR involved in Purkinje cell degeneration. The

classic Purkinje cell degeneration pattern starts at anterior and posterior zones and follows by central and nodular zones. The cerebellar patterning is made of Purkinje cells scaffold arrangement and afferents and efferents are aligned in a zone and stripe pattern. In Acp2 mutant mice with same pattern of cerebellar Purkinje cell degeneration (Purkinje cell loss), we investigate and explore any alterations in the pattern expression of P75NTR beside other cerebellar cortex compartmentation well-known markers like zebrin II, HSP25. The expression of the p75NTR when evaluated in the early postnatal days, it was shown strong uniform immunoreactivity in Purkinje cells and weak immunoreactivity in cerebellar nuclei. In the transverse section of the adult cerebellum, the immunoreactivity pattern of HSP25is strongly present in Purkinje cells of the central and nodular zones, but not in the anterior and posterior zones. P75NTR expressed in the same pattern as HSP25 zone and stripe phenotype in adult cerebellum in both control and Acp2 mutant mice. Interestingly, the presence of p75NTR stripes maintains in lobule VII of adult cerebellum.

5.1 Introduction

Lysosomal acid phosphatase 2 (Acp2) is a key enzyme in cerebellar development (Bailey et al., 2013b, Bailey et al., 2014b, Mannan et al., 2004), and it is implicated in a variety of neurological disorders including progressive supranuclear palsy (Melquist et al., 2007) and juvenile neuronal ceroid lipofuscinosis (a disease caused by mutations in the lysosomal protein ceroid-lipofuscinosis neuronal 3 (CLN3), which is also called Batten disease) (Pohl et al., 2007). Acp2 hydrolyzes orthophosphoric monoesters into alcohol and phosphate. Mounting evidence indicates that a spontaneous autosomal recessive mutation in Acp2 is responsible for occurrence of a neurocutaneous disorder that is correlated with abnormal skin and cerebellar development, and is also called *naked-ataxia* (*nax)* mice (Mannan et al., 2004, Bailey et al., 2014b). In addition to the *nax* mouse, several studies have used the Acp2 transgenic animal model to investigate the role of Acp2 deficiency in development of nervous system, particularly in the cerebellum (Bailey et al., 2014b, Mannan et al., 2004, Saftig et al., 1997, Makrypidi et al., 2012).

Based on the differential gene expression, the cerebellum has been compartmentalized into four transverse zones including: 1) the anterior zone (AZ: lobules I-V); 2) the central zone (CZ: lobules VI-VII: with two sub-zones – see (Marzban et al., 2008b, Sawada et al., 2008)); 3) the posterior zone (PZ: lobules VIII + dorsal IX); and 4) the nodular zone (NZ: ventral lobule IX + lobule X) (e.g., (Ozol et al., 1999b, Bailey et al., 2013b, Marzban and Hawkes, 2011)). In addition, each zone is subdivided mediolaterally into parasagittal stripes that are symmetrical about the midline (Apps and Hawkes, 2009, Ozol et al., 1999b). Much of the cerebellar patterning seems to have been built on the Purkinje cell (Pc) scaffold (Consalez and Hawkes, 2012) because cerebellar cell types, functions and their afferent and efferent roles are aligned in a zone and stripe pattern (Chung et al., 2009, Marzban et al., 2010, Marzban et al., 2007).

Our laboratory has recently revealed that Acp2 not only plays a pivotal role in development of the cerebellum through expression in the caudal mesencephalon and diffusely in the developing cerebellum, but also in its expression in the adult mouse cerebellum by a subset of Pcs that are aligned in the zebrin II zone and stripe pattern (Bailey et al., 2013b). Another important finding using the *nax* mouse was that the pattern of the gene expression in the cerebellum is altered particularly in the AZ and CZ (Bailey et al., 2014b). The density and population of Pcs decreased significantly in response to unknown cell death mechanism with aging in the *nax* cerebellum. We have previously shown that probably Pcs death in *nax* cerebellum are not associated with the classic apoptosis signaling pathway (Bailey et al., 2014b), such as observed in the Pc degeneration (pcd) mouse (Chakrabarti et al., 2009, Fayaz et al., 2014).

On the other hand, neurotrophins are highly involved in processes related to neuronal survival, development and function (Huang and Reichardt, 2001, Park and Poo, 2013). Neurotrophins have been reported to regulate neuronal apoptosis, a process that is critical in the developing brain and also in a variety of neurodegenerative diseases (Yuan and Yankner, 2000). During development, nerve growth factor (NGF) mediates a variety of neuronal functions through activation of the p75NTR (Ichim et al., 2012b). Depending on the physiological, pathophysiological or developmental timing of the brain, it has been proposed that p75NTR plays a dual role in cell survival or death (Mamidipudi and Wooten, 2002, Florez-McClure et al., 2004).

In pathological conditions, upregulation of p75NTR and replacement of this receptor is the key in controlling the numerous processes that are necessary for nervous system recovery (Meeker and Williams, 2015). Currently, p75NTR is an important potential target for pharmacological control of neurotrophin activity (Meeker and Williams, 2015). In this respect,

we aimed to examine whether p75NTR is altered with progressive Pc loss in a *nax* model of neurodevelopmental and neurodegenerative diseases.

5.2 Materials and Methods

Animal maintenance and tissue processing

Animal procedures conformed to institutional regulations and the *Guide to the Care and Use of Experimental Animals* from the Canadian Council for Animal Care (CCAC). This study was approved by the University of Manitoba Animal Care Committee (ACC). All experiments conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and the animals were treated in a humane manner.

Nax mutant embryos were obtained from the Institute of Human Genetics in the University Medical Center, Georg-August University, Goettingen, Germany. A colony was established in the Genetic Model Center at the University of Manitoba by breeding mice (C57BL/6) heterozygous for the *nax* mutation (the homozygote:heterozygote:wild type ratio was 25%:50%:25%, respectively). Animals were maintained at room temperature and relative humidity (18–20°C, 50–60%) on a 12-h light-dark cycle with free access to food and water.

Phenotypically, *nax* mutant mice were easily distinguished from their littermate controls based on the delayed appearance or lack of hair over the entire body, smaller stature and ataxia. To confirm genotypes, PCR was performed according to Mannan et al. (2004), using the following primers: Acp4F (GCACTCTGTGCCTTCTCCAT) and Acp4R (CTGGGAGATTTGGGCAACTA). Samples were prepared as we previously described (Bailey and Rahimi Balaei, 2014). Briefly, adult and postnatal developing mice from P4 to P22 (wild

type (wt); n= 11 and *nax*; n= 11) were transcardially perfused and brains postfixed in 4% paraformaldehyde. Then, cerebella were cryoprotected in sucrose series and optimal cutting temperature to take section at 30 μm in transverse and sagittal plane.

Antibodies

Two anti-calbindin antibodies were used: Rabbit polyclonal anti-calbindin D-28K antiserum (anti-CaBP, diluted 1:1000, Swant Inc. Bellinzona, Switzerland) was produced against recombinant rat calbindin D-28K. In the cerebellum, CaBP is exclusively expressed in Pcs (e.g. (Tano et al., 1992, Baimbridge and Miller, 1982)). Mouse monoclonal anti-calbindin was (anti-CaBP, diluted 1:1000, Swant Inc. Bellinzona, Switzerland) raised against chicken calbindin D-28K was used. Immunohistochemistry yielded Pc-specific staining identical to that previously reported (e.g. (De Camilli et al., 1984).

For the p75 neurotrophin receptor (p75NTR), rabbit polyclonal antibody, was purchased from Cell Signaling Technology, Massachusetts, USA (diluted 1:1000). p75NTR is expressed in some cerebellar Pcs (Lotta et al., 2014).

For heat shock protein 25, anti-small heat shock protein 25 (HSP25) is a rabbit polyclonal antibody obtained from StressGen, Victoria BC, Canada (diluted 1:1000). Hsp25 is expressed in a subset of Pcs (for details see (Armstrong et al., 2000a, Bailey et al., 2014b).

For the paired box protein, anti-paired box protein (Pax6) was used for the mouse monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Department of Biology, USA) and it was diluted 1:1000. This transcription factor is expressed in developing cerebellar granule cells (Engelkamp et al., 1999a).

Section Immunohistochemistry

Peroxidase and double-label fluorescent immunohistochemistry were performed as described previously (Marzban et al., 2008b, Bailey et al., 2013b). Briefly, for peroxidase immunohistochemistry, tissue sections were washed with PBS and endogenous peroxidase activity was blocked using 0.3% H2O2. Sections were incubated in blocking solution then in primary antibody for overnight at room temperature. After washing with PBS, sections were incubated in appropriate secondary antibodies at room temperature then developed by diaminobenzidine (DAB) as the chromogen. For negative control, sections were processed same as above but addition of primary antibody was replaced by a suitable IgG isotype. Tissue was washed with PBS again and Peroxidase activity was revealed by using DAB. For anti-HSP25 immunohistochemistry, biotinylated secondary antibody was used.

For double immunofluorescence sections were co-incubated in primary antibodies overnight at room temperature and then incubated in Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG for 1 hr at room temperature. Sections were then washed in PBS and coverslipped with Fluorsave.

Western blotting

Western blot analysis of the cerebellum was performed using a conventional protocol (e.g. (Bailey et al., 2013b). To measure the expression of p75NTR, cerebellar tissue was homogenized at different ages at postnatal (P) day P5, 9 and 18 and protein concentrations were measured using a BCA kit. Diluted samples in loading buffer electrophoresed and transferred to an Immobilon-P transfer membrane (Millipore, Mississauga, ON). For immunostaining, membranes were blocked for one hour in 5% skim milk in TBS + 0.1% Triton X-100 (PBST). Membranes

were incubated with primary antibody (Rabit polyclonal anti-p75NTR was diluted in PBST) at 4°C for 18 hours with gentle agitation. Membranes were incubated for 1 hour with HRP conjugated goat anti-rabbit IgG diluted 1:6000 in TBST. Binding was detected using the Enhanced Chemiluminescence (ECL) protocol on Scientific Imaging Film.

Figure preparation

For bright field microscopy, a Ziess M-2 microscope was used and images were captured using Zen software. For fluorescence microscopy, a Ziess Lumar.V12 stereomicroscope was used to capture images of entire cerebellar sections using AxioVision 4 software. For high magnification, fluorescence microscopy a Ziess Z2 Imager with Zen software and a Zeiss LSM 700 confocal microscope with Zen software were used to obtain images. Images were cropped, corrected for brightness and contrast, and assembled into montages using Adobe Photoshop CS5 Version 12.

Statistical methods

Western blot data are presented as the mean ± standard error of mean (SEM) and they were analyzed using Kruskal-Wallis test followed by Dunnett`s multiple comparison test using Mann-Whitney test. We considered $p > 0.05$ as non-significant result. All statistical analysis was performed using SPSS software for windows (Version 18 SPSS Inc. Chicago, IL, USA).

5.3 Results

Patterned expression of p75NTR in adult mouse cerebellum

A transverse section through the cortex of adult mouse cerebellum showed that p75NTR is strongly (arrow) or weakly (arrowhead) expressed in Pc somata and dendrites in the molecular layer (Fig. 5.1 A). To confirm p75NTR expression in Pcs we performed double immunostaining with CaBP (a specific marker for Pcs) and showed p75NTR localization in Pcs (Fig. 5.1 B). p75NTR expression is restricted to a subset of Pcs in the cerebellar cortex that alternate with p75NTR immuno-negative Pcs (Fig 5.1 C-E). Low magnification of transverse sections through the mouse cerebellum showed that p75NTR express in parasagittal strips pattern and symmetrical about the midline (Fig. 5.1 F). The pattern immunoreactivity of p75NTR is strongly present in the CZ (lobule VI/VII) and NZ (IXb/X), but not in the AZ (I-V) and PZ (VIII/IXa) of the adult cerebellum (Fig. 5.1 F). p75NTR expression is similar to the pattern of HSP25 expression in mouse cerebellum that is localized to the CZ (Fig 5.1 G) and NZ (e.g. (Bailey et al., 2014b)).

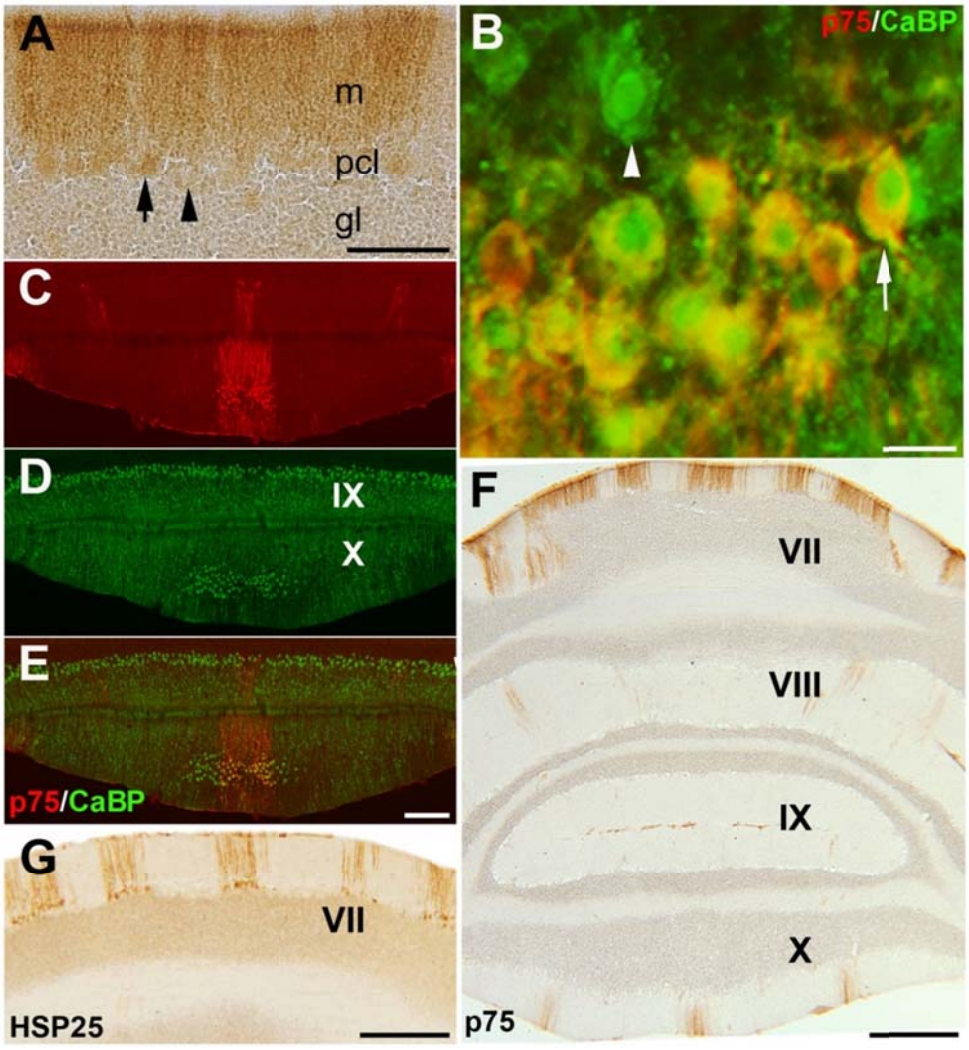

Figure 5. 1 Frontal sections of an adult mouse cerebellum immunostained with p75NTR, CaBP and HSP25 at P28.

The lobules are indicated by Roman numerals. A-B) The cerebellar cortex immunoperoxidase stained by p75NTR shows that the Purkinje cell somata in the Purkinje cell layer (Pcl) and dendrites in the molecular layer (ml) are immunoreactive (arrow; black in A, and white in B). Some Purkinje cell somata expressing p75NTR are weak (A, arrowhead) or absent (B, arrowhead). C-E) Double immunostaining of a frontal section through lobule IX and X using p75NTR (red) and CaBP (green). Stripes of immunoreactive Purkinje cells are clear in the

vermis. F) Immunostaining of a frontal section through the cerebellum using p75NTR. Immunoreactivity is not present in the AZ and PZ. Stripes of immunoreactive Purkinje cells are clear in the vermis of the CZ (VII) and NZ (X). G) Immunostaining of a frontal section through the lobule VII using HSP25. Stripes of immunoreactive Purkinje cells are clear in the vermis of the CZ. Scale bars: A= 100 μm; B= 20 μm; E= 250μm (applies to C-E); F= 500 μm; G= 250 μm

Patterned expression of p75NTR in wt and nax cerebellum at P4 and P6

p75NTR expression was studied in midsagittal and frontal sections of *nax* and wt sibling cerebellum to determine distribution of p75NTR in developing cerebellar cortex at P4 and P6. Double immunohistochemistry with CaBP revealed that p75NTR was widely expressed in midsagittal sections throughout the cerebellar cortex of wt and *nax* mice at P4 (Fig. 5.2 A and 5.2 D). At a higher magnification, it is shown that p75NTR expression is localized in the developing Pc layer in the wt sibling (Fig. 5.2 B) and *nax* (Fig. 5.2 E) cerebellar cortex , with strong expression in the Pc membrane and weakly in the cytoplasm (Fig. 5.5 G-I)). In addition, p75NTR is expressed in the area superficial to the developing Pc layer, at the location of the external germinal zone (EGZ) (Fig. 5.2 B, E). Double staining with Pax6, a marker for granule cell precursors confirmed that p75NTR is expressed in the EGZ (Fig. 5.2 C, F). A sagittal section through the cerebellar cortex immunoperoxidase stained by p75NTR shows immunoreactivity of the Pc in wt (J) and *nax* (K), in which Pc are multilayered in molecular layer.

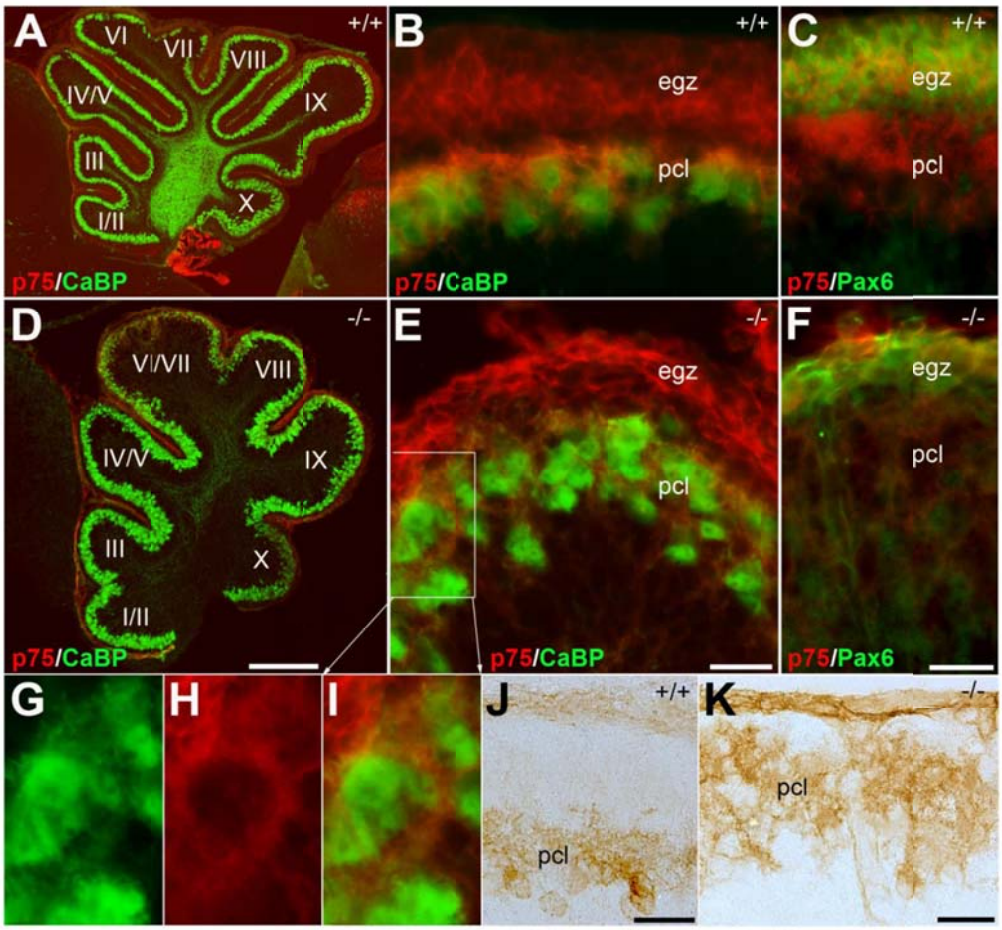

Figure 5. 2 Sagittal sections of P4 wt (A-C) and *nax* (D-I) and sagittal sections of P6 wt (J) and nax (K) cerebella immunostained with p75NTR, CaBP and Pax6.

The lobules are indicated by Roman numerals. **A-C)** The wt cerebellum shows normal lobules (A) with expression of p75NTR (red) in the entire EGZ and dispersing through the Pc layers. A higher magnification of "A" shown in "B" and Pax6 immunostaining is indicated EGZ in "C". **D-F**) The *nax* cerebellum shows a small cerebellum with underdeveloped lobulation (B). p75NTR expression (red) is present in the underdeveloped EGZ and in the developing Pcl (B). Immunostaining with Pax6 shows the thin layer of the EGZ in the *nax* cerebellum (F). **G-I**) Magnified views of white box in E showing CaBP (G), p75NTR (H), and merged image (I). **J-K**) The sagittal sections immunoperoxidase stained by p75NTR shows immunoreactivity of the Pc in wt (J) and *nax* (K) cerebella. Abbreviations: EGZ, external germinal zone; Pcl, Purkinje

cell layer. Scale bars: D= 500 µm (applies to A and D); E= 50 µm (applies to B and E); F= 40µm (applies to C and F); J= 50 µm; K= 50 µm.

To understand whether p75NTR expression is uniformed or in stripes pattern to study the possible alteration in *nax* in comparison with wt sibling, the early postnatal cerebellar cortex immunoperoxidase stained by p75NTR at P6 (Fig. 5.3). P75NTR is expressed in cerebellar cortex and weakly in cerebellar nuclei probably due to Pc terminal axon in cerebellar nuclei region (Fig. 5.3 A). The pattern expression of p75NTR is uniform in entire cerebellar cortex (Fig. 5.3 A) and clearly in Pc layer and external germinal zone, but not in granule cell layer (Fig. 5.3 B). Despite of underdeveloped *nax* cerebellar vermis and atypical lobulation the pattern is comparable to wt sibiling (Fig. 5.3 C). The p75NTR is expressed uniformly in cortex of *nax* cerebellum in which multilayer Pc are invaded to molecular layer accompany with thin or lack of external germinal zone and granule cells (Fig. 5.3 D).

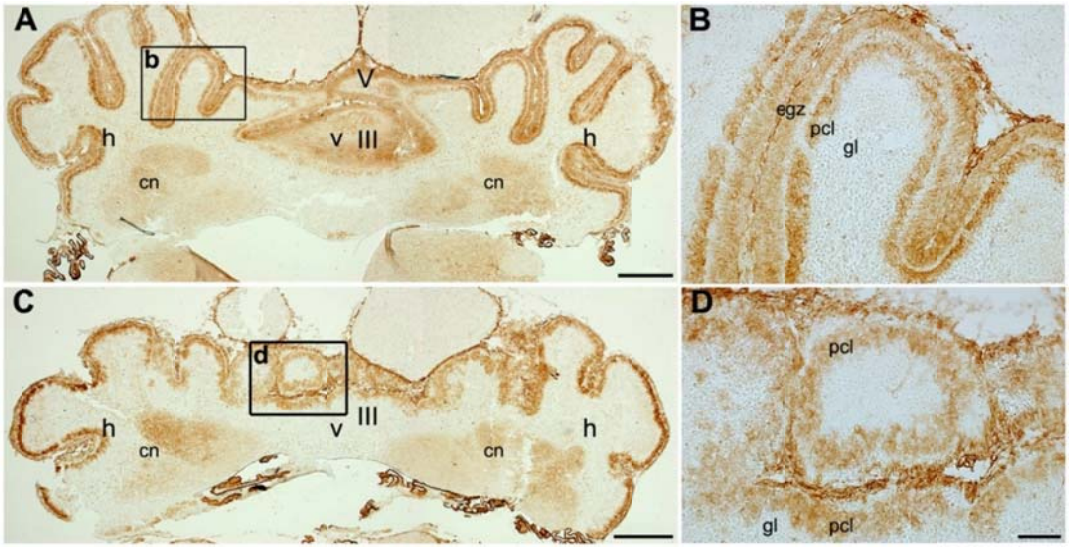

Figure 5. 3 Frontal sections through the cerebellum of wt (A-B) and *nax* (C-D) at P6 immunoperoxidase stained with p75NTR.

A-B) The wt cerebellum shows normal vermis (v) and hemisphere (h) lobulation (A) with uniform expression of p75NTR in the entire cerebellar cortex. A higher magnification of black box (b) in "A" indicate localization of p75NTR expression in Pcl and EGZ. C-D) The underdeveloped *nax* cerebellum shows uniform p75NTR expression in putative vermis and in the hemisphere. D) Magnified views of black box (d) in "C" showing p75NTR in multilayer Pc and thin or lack of EGZ. The p75NTR expression shows the outline of cerebellar nuclei (cn) both in wt (A) and *nax* (C) sections. Abbreviations: EGZ, external germinal zone; Pcl, Purkinje cell layer. Scale bars: A and C= 500 μm; D= 100 μm (applies to B and D).

Overall, the pattern of p75NTR expression was normal at P4/P6 in *nax* mice, despite the small size of the cerebellum, underdeveloped lobulation and corticogenesis with an abnormal Pc multilayer and a thin layer, or lack of EGZ.

Patterned expression of p75NTR in wt and nax at P22

Cell death is prominent in the *nax* cerebellum (Bailey et al., 2014b) and p75NTR is suggested to be involved in Pc degeneration (Florez-McClure et al., 2004). To determine whether p75NTR pattern expression was altered in *nax* cerebellum that undergoes severe cerebellar cell death, we examined p75NTR pattern expression in *nax* and wt siblings and compared it with HSP25 at P22 (Fig. 5.4). Double immunostaining with p75NTR and CaBP through the CZ and NZ of the *nax* mutant cerebellum revealed an array of parasagittal stripes (Fig. 5.4 A), which was shown at a higher magnification of lobule VI (Fig. 5.4 B). P75NTR expression in CZ and NZ was also compared with HSP25 expression in the CZ and NZ in the *nax* cerebellum. Because both p75NTR and HSP25 are rabbit polyclonal antibodies, double staining was not performed. Consequently, we used serial section of the *nax* cerebellum to compare the p75NTR and HSP25

expression pattern. P75NTR was similar to that of HSP25 in the CZ and NZ (Fig 5.4 C) and a higher magnification of CZ is shown in Fig. 5.4 D. Double immunofluorescence staining with p75NTR and CaBP revealed the presence of p75NTR stripes in lobule VII of wt siblings (Fig. 5.4 E).

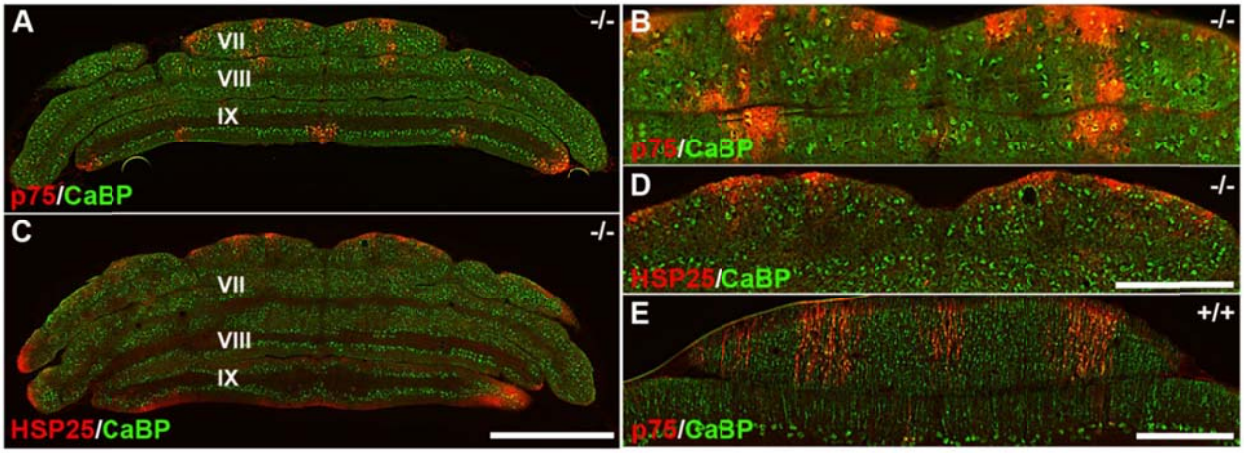

Figure 5. 4 Transverse sections of *nax* (A-D) and wt sibling cerebella at P21 immunostained with p75NTR, CaBP and HSP25.

A,B) p75NTR immunostaining shows an array of parasagittal stripes in the CZ and NZ of the *nax* cerebellum, with high magnification of the CZ shown in **B**. **C-D**) HSP25 immunostaining of the *nax* cerebellum shows parasagittal stripes in the CZ and NZ, **D** is a higher magnification of **C**. **E**) At a high magnification, p75NTR expression is shown in the CZ of the wt sibling. Scale bars: C= 1 mm (applies to A and C); D= 500 µm (applies to B and D); E= 250µm.

Protein expression of p75NTR in wt and nax cerebellum

To determine whether the protein expression of the p75NTR is altered in *nax* and wt siblings, we used western blot at P5, P9 and P18. Western blot analysis indicates that p75NTR protein expression gradually decreases as development proceeds in both *nax* and wt siblings. However, despite the apparent higher expression of p75NTR in the *nax* cerebellum or probably delayed

down regulation of p75NTR protein expression, there is no significant difference between *nax* and wt sibling cerebellum at any of these ages (Fig. 5.5 A, B) (p>0.05).

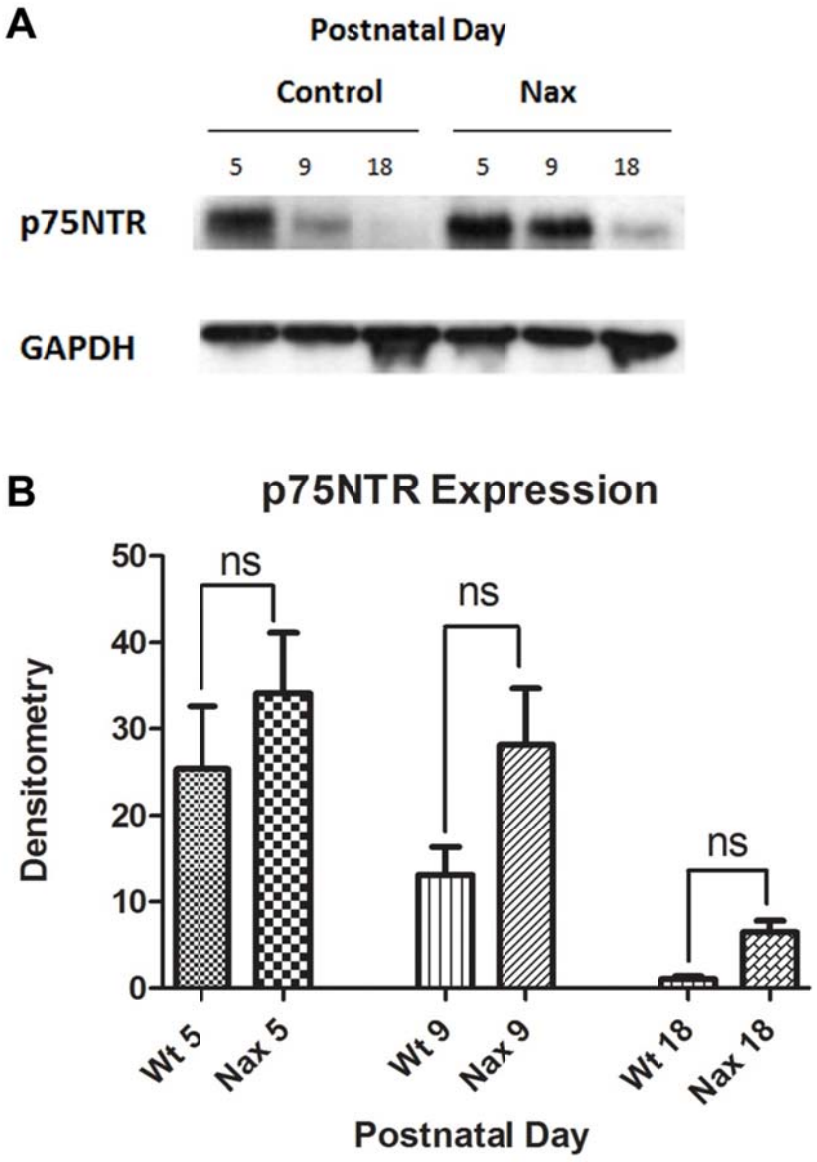

Figure 5. 5 Western blot analysis of p75NTR expression during cerebellar development at P5, P9 and P18.

A) Immunoblots of total cell lysate from wt sibling and *nax* mouse cerebellum indicate significant down-regulation in the wt sibling and also in the *nax* cerebellum from early postnatal

development to P18. However, there is no significant difference in the p75NTR expression between the wt and *nax* cerebellum, despite the higher p75NTR expression in *nax* compared with wt. (p>0.05). Protein loading was confirmed using GAPDH (n=3). **B)** The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using using Kruskal-Wallis test followed by Dunnett`s multiple comparison test using Mann-Whitney test.

5.4 Discussion

In this study, we examined p75NTR expression in postnatal *nax* mutant mice cerebellum to determine the pattern and protein expression to identify whether abundant Pc degeneration in *nax* cerebellum is associated with alteration of the p75NTR expression. The p75NTR expression is localized in the EGZ and Pc layer in early postnatal development, while it is patterned in parasagittal stripes in the CZ and NZ of the adult mouse cerebellum, similar to HSP25 pattern expression. Although the *nax* cerebellum has severe abnormalities and Pc degeneration (Bailey et al., 2014b) there seems to be no alteration in the p75NTR pattern and protein expression in the *nax* cerebellum compared with the wt siblings.

The cerebellar compartmentation has a fundamental cytoarchitecture organization in which gene expression, function and pathology are aligned in zone and stripe patterns (Bailey et al., 2013b, Bailey et al., 2014b, Marzban et al., 2007, Armstrong et al., 2000a, Chung et al., 2009). The pattern of gene expression in different cerebellar lobules indicates different insult vulnerability levels. Pc death is a topographically-regulated process rather than a random sequence of events in the cerebellum (Sarna and Hawkes, 2003a) and it is a complex progression in neurodevelopmental disorders (Sarna and Hawkes, 2003a, Sarna et al., 2003a, Bailey et al.,

2014b). In the cerebellum, several studies involving mutant mice have shown that there is a general spatio-temporal pattern of Pc degeneration. These models include mouse models of Niemann-Pick type C disease (Sarna et al., 2003a), the lurcher mutant mouse cerebellum (Duffin et al., 2010) and the ataxic sticky mouse cerebellum (Sarna and Hawkes, 2011). In most cases, specific Pc populations, which are located within the AZ and PZ, degenerate earlier than those from the CZ and NZ. It has been shown that Pc degeneration usually appears in a strip-like pattern that corresponds to a specific region within the cerebellum (Sarna and Hawkes, 2003a). In murine models of Niemann-Pick disease type C, Pc loss first occurs within the AZ and PZ, and then progresses to the CZ Pcs, in which HSP25 is expressed in pattern parasagittal stripes. However, the Pcs that express HSP25 are more resistant to degeneration than those lacking this protein (Sarna et al., 2003a, Duffin et al., 2010).

We have shown that despite abundant cleaved caspase-3 immuno-positive cells in the *nax* cerebellar cortex, none of these cleaved caspase-3^{+} cells were identified as Pcs. This suggests that classic apoptosis may not be responsible for Pc death in the *nax* mutant (Bailey et al., 2014b). Given that the Pc degeneration is usually reflecting the foundamental architecture in cerebellum, in *nax* cerebellum either due to Pcs degenartion or developemental impairment, the number of zebrin II stripes is decreased in AZ.

Numerous genes are expressed uniformly or are entirely immune-negative in the CZ and NZ of mouse cerebellum (e.g. (Bailey et al., 2013b, Marzban et al., 2007, Sarna et al., 2006a, Marzban et al., 2003c, Marzban et al., 2003a)). In contrast, a few genes such as HSP25 are expressed in pattern parasagittal stripes in the CZ and NZ (Bailey et al., 2014b, Armstrong et al., 2000a). Surprisingly, in this study p75NTR pattern expression seems to be aligned with HSP25 expression pattern in the CZ and NZ of the adult mouse cerebellum. It has been shown that a

subset of Pcs that are HSP25 immuno-negative are more vulnerable than HSP25 immuno-positive Pcs in the CZ and NZ, which suggests that HSP25 may have a protective role in these subsets of Pcs (Armstrong et al., 2005, Bailey et al., 2014b). HSP25 function is unclear in the cerebellum, but in non-neuronal cell lines, it is a molecular chaperone (e.g. (Jakob et al., 1993)) that regulates actin filament organization and stabilization during oxidative stress (e.g. (Lavoie et al., 1993)), regulates anti-oxidative activity (Mehlen et al., 1996), protects cells and increases cell survival (Sarna and Hawkes, 2003a). Although the function and role of the p75NTR expression patterns in parasagittal stripes in the CZ and NZ remain to be determined, this may indicate that p75NTR in adult cerebellum accompanied by HSP25 plays a role in protection of this subset of Pcs from death. This speculation is promising because Pcs in these stripes are resistant to death in numerous neurodegenerative mutant cerebella, but there is no significant difference in the p75NTR expression pattern between the *nax* and wt sibling cerebella, even though there are abundant Pc death in the severed abnormal *nax* mutant cerebellum. Large Pc size, extensive connectivity and high metabolic demand lead to high vulnerability of Pcs and these neurons are specifically lost in various neurodegenerative diseases, but the molecular mechanisms underlying Pc death remain poorly understood.

It has been shown that NGF likely interacts with the tyrosine kinase receptor p75NTR (Roux and Barker, 2002).In addition, it is believed that all neurons or normal cells are naturally committed to die unless a survival factor such neurotrophin blocks this death through its receptors (Ichim et al., 2012b). However, neurotrophin withdrawal is associated with an active signal of cell death that is induced by unbound dependence receptors (Ichim et al., 2012b). These results are in agreement with recent studies indicating that small, non-peptide p75NTR ligands can selectively modify pro-survival and repair functions (Meeker and Williams, 2015). Given

that there is no caspase-3 activation in degenerating Pcs in the *nax* cerebellum and this may imply that Pc death occurs through other death mechanism pathways (Bailey et al., 2014b). Recently, it has been suggested that autophagy is involved in Pc death as a result of trophic factor deprivation through p75NTR, which is a possible mediator of the Pcs death (Florez-McClure et al., 2004). Therefore, upregulation and/or downregulation or pattern and protein expression changes in p75NTR are expected under pathological conditions such as in the *nax* cerebellum because of dysregulation of pathways for numerous molecular processes and the necessity of reactions for neuronal death protection or recovery.

The pattern of p75NTR expression between the *nax* and wt sibling cerebellum was similar in immunohistochemistry and its protein expression was not significantly different during postnatal cerebellar development. Given that the HSP25 expression pattern plays a protective role in Pc death in the cerebellum of neurodegenerative mutant mice, p75NTR expression is expected to be comparable. Indeed, p75NTR protein expression in the *nax* cerebellum align with this speculation, and p75NTR expression is in the parasagittal stripe pattern in the CZ and NZ in *nax* and wt siblings, and it seems indistinguishable to the HSP25 pattern expression.. p75NTR protein expression in western blot analysis shows some upregulation, but these differences are not statistically significant between *nax* and wt. However, severe abnormality occurs in the AZ accompanied by Pc death and our data shows that the majority of Pcs in the AZ do not express the p75NTR in adult cerebellum.More experiments are required to explore the complex functions of p75NTR expression in the cerebellum and possible protective roles that may be involved in Pc death in the *nax* cerebellum. The complexity of Pc death indicated that these large cells use different mechanisms to survive and protect their function, and therefore, different molecular pathways may trigger cell death in Pcs in pathological conditions such as the *nax* cerebellum.

5.5 Conclusion

We investigated p75NTR pattern and protein expression in the *nax* mouse with severe cerebellar anomalies and Pc degeneration and in the wt sibling cerebellum. p75NTR expression is similar to the HSP25 expression pattern in the adult mouse cerebellum. In the *nax* cerebellum, the p75NTR pattern is similar to that of HSP25 and there is no significant difference between *nax* and wt siblings in p75NTR protein expression.

CHAPTER SIX: DISCUSSION

6.1 Overview

The cerebellum is an important structure in the central nervous system that controls and regulates motor and non-motor functions like cognition, emotion, and language processing. Although the cytoarchitecture of the cerebellum with few cell types looks simple, but there is an elaborate control during cerebellar neurogenesis. The correct number of cerebellar cells, migration, positioning, and circuit formation are important while they receive different afferents and provide efferent outcomes to refine some of brain functions. Cerebellar development is very complicated as it starts at embryonic stage and ends in postnatal days. The origin of cells within the cerebellar primordium are outlined at the early embryogenesis stages, but cerebellar maturation as a whole continues well into postnatal life. The protracted development of the cerebellum makes it susceptible to a variety of malformations that range from vermis agenesis to neocerebellar aplasia. These anomalies usually accompany cerebellar nuclei defects such as a fragmented dentate nucleus in *Joubert syndrome* (Joubert et al., 1969, Manto et al., 2012), and segmental loss of the dentate nuclei in *Pontocerebellar hypoplasia*, (Gardner et al., 2001, Manto et al., 2012). The cerebellar nuclei defect in *Rhombencephalosynapsis* is a common feature in several cerebellar disorders and it is most commonly associated with GLHS (Gomez-Lopez-Hernandez Syndrome, a neurocutaneous disorder, also known as cerebellotrigeminal-dermal dysplasia) (Ishak et al., 2012, Fernandez-Jaen et al., 2009). In this developmental defect, the dentate nuclei and cerebellar hemispheres are fused in the midline due to absence of a cerebellar vermis (Ishak et al., 2012). Cerebellar nuclei connectivity defects are present in several types of ataxia (abnormal and uncoordinated movements) such as Friedreich's ataxia, spinocerebellar

ataxia type 3 (SCA3), and SCA6 (Stefanescu et al., 2015). As there are around 30 different cerebellar ataxia, the role of cerebellar nuclei connectivity defects need more attention in future studies. In addition to the major structural defects, more subtle conditions such as autism, ataxia, neuropsychiatric disorders, which are because of minor changes in neuronal formation, migration and connectivity. For instance, in patients with autism spectrum disorder (ASD) , common findings in the cerebellum of this neurodevelopmental disorder are a reduction of the Purkinje cells density in the cerebellar cortex and loss of neurons in cerebellar nuclei (La Malfa et al., 2004, Palmen et al., 2004, Manto et al., 2012). Any defects during this whole process would affect the individual skill performances. For preventing these defects or designing effective therapeutics, understanding cerebellar development would be of interest. During cerebellar neurogenesis, two germinal zones are involved which are located in cerebellar primordium and in this thesis I describe an external germinal zone, mesencephalon, which produce a subset of cerebellar nuclei neurons prior to other cerebellar neurons. This group of neurons expresses SNCA, Otx2 and p75NTR. The origin and role of these cells were investigated during cerebellar development. The role of SNCA during embryonic and postnatal stage was explored in the mice with the absence of this transcription factor. The role of p75NTR as neural crest marker was evaluated in normal and a mouse model with cerebellar defect (*nax* mutant mice). In this chapter, I will highlight and summarize my findings and will explain about the limitations I faced for each of these studies and will enclose with the future directions.

6.2 Early subset of cerebellar nuclei neurons derived from mesencephalon in mice

In this study, I investigated the mesencephalic-derived cerebellar nuclei neurons during early cerebellar development. Recent studies indicated that most cerebellar nuclei projection

neurons originate from the rhombic lip at E9 in mice and sequentially express *Pax6*, *Tbr2*, and *Tbr1/Lmx1a* genes and are present in nuclear transitory zone (NTZ) at E10 (Ben-Arie et al., 1997a, Fink et al., 2006c, Machold and Fishell, 2005). We showed that a novel subset of cerebellar nuclei neurons ($SNCA^+/Otx2^+$ neurons) run from the dorsal mesencephalon and cross the isthmus to the NTZ as early as E9 (prior to the rhombic lip derived cells). This suggests the presence of a new germinal zone during cerebellar neurogenesis. Further, these cells are a group of differentiating neurons (NAA^+) and express $Otx2^+/p75NTR^+/Wnt1^+/TrkA^+$, suggesting that this subset of CN neurons is possibly derived from the mesencephalic neural crest population. In the mesencephalon, the mesencephalic nucleus of the trigeminal nerve is the large sensory neurons which are derived from the neural crest (Narayanan and Narayanan, 1978a, Lumsden et al., 1991). Beside neural crest markers, this was checked by administration of DiI to the mesencephalon and shown that these mesencephalic derived cells form a subpopulation of CN neurons that likely predates all neurogenesis in the cerebellar primordium. This novel subset of cerebellar nuclei neurons is accompanied by the nerve fibers that express activated caspase-3, restricted within the cerebellar primordium core and indicate another role of cleaved caspase-3 as axonal guidance/survival. Our results indicate that this subset of the cerebellar nuclei neurons is present until E15 (also at E18, data has not shown) at the cerebellar primordium core while expressing Otx2, SNCA is down regulated. The fibers terminate at the midway point of the rostrocaudal subpial surface (these fibers divide cerebellar primordium to anterior and posterior parts) are probably originated from early cerebellar nuclei neurons or from pioneer axons that originate from the trigeminal nerve and may use as substrate for $Lmx1a^+$ (rhombic lip derived) cerebellar nuclei projection neurons. This suggests that the mesencephalic derived

$SNCA^+/Otx2^+/p75NTR^+$ combination of neurons/fibers possibly plays a role as signaling center during cerebellar development.

Conclusion

My study suggests that a subset of cerebellar nuclei neurons that are $SNCA^+/Otx2^+/p75ntr^+$ originates from the mesencephalic neural crest, which is a possible new germinal zone for cerebellar development. We showed that this subset of cerebellar nuclei neurons accompanies nerve fibers that are present at the core of cerebellar primordium and terminates at the midway of the rostrocaudal subpial surface. This suggests that they play a role as a secondary organizer that may act as a signaling center. This center probably directs rhombic-lip derived cells to the NTZ and also organizes the cerebellum into anterior and posterior divisions at the level of lobule VI/VII.

6.3 Cerebellar cortex patterning in prostatic acid phosphatase mutant mouse with absence of the α-synuclein gene

In this study, to investigate the role of SNCA expression during embryonic and adult cerebellum (in our previous study we have shown a subset of mesencephalic derived neurons with condensed expression of SNCA in NTZ of early developing cerebellum), we have shown that despite the importance of the expression of PAP and SNCA during development, double knock-out mice for these genes (*Pap*/*Snca KO*) display normal brain morphology, including stripes and zone patterns of the cerebellar cortex architecture and NTZ cells are still there when labeled by p75NTR. Neuronal proliferation/differentiation/survival is regulated by P75NTR that are mainly expressed during early development by neural crest cells (Jiang et al., 2008, Bernabeu and Longo, 2010b, Dechant and Barde, 2002a).

There is a fundamental cytoarchitecture organization in the cerebellar compartmentation and each gene is expressed, functioned and aligned in the pattern of zones and stripes and is the best way to study any Purkinje cell degeneration between different lobules (Bailey et al., 2014a, Rahimi-Balaei et al., 2018b, Rahimi-Balaei et al., 2015b). Well-known Purkinje cell markers were used: Calb1, zebrin II, P75NTR (our finding in chapter five) and HSP25 and confirmed that there is no difference between knockout and WT control cerebellum with normal appearance of the stripe and zone patterns of these genes (Calb1, zebrin II, P75NTR and HSP25). All in all were in support of the normal cerebellar compartmentation and morphology in dual *Pap / Snca KO* mice.

Conclusion

The most obvious finding to emerge from this study is that *Pap* and *Snca* dual KO mice have normal cerebellar morphology. These findings will be of interest to clinicians who are working on Parkinson's disease, Alzheimer's disease or other synucleinopathies.

6.4 The neurotrophin receptor p75 expression in the cerebellum of the lysosomal acid phosphatase 2 mutant mouse (*nax* - naked-ataxia mutant mouse)

We have shown that P75NTR pattern and protein expression in the postnatal cerebellar section is comparable with HSP25 expression (Rahimi Balaei et al., 2016).

The expression of p75NTR was shown in NTZ and fibers terminate at the midway point of the rostrocaudal subpial surface during cerebellar development. The expression pattern of p75NTR in control WT and *nax* mutant mice of postnatal cerebellum was investigated. P75NTR

has a role in Neuronal proliferation/differentiation/survival. *Nax* mutant mice are with abundant Pc degeneration, so we explore any association with alteration of the p75NTR expression. The p75NTR expression is localized in the EGZ and Pc layer in early postnatal development, while it is patterned in parasagittal stripes in the CZ and NZ of the adult mouse cerebellum, similar to HSP25 pattern expression. Although the *nax* cerebellum has severe abnormalities and Pc degeneration there seems to be no alteration in the p75NTR pattern and protein expression in the *nax* cerebellum compared with the wt siblings. $HSP25^{+}$ Purkinje cells are more resistant to degeneration than those lacking this protein and zebrin II^{+} Purkinje cells in anterior zone ($p75NTR^{-}$ area) are decreased in *nax* cerebellum. The expression of p75NTR in Purkinje cells aligned with HSP25 and may involve in protection of this subset of Pcs from death in CZ and NZ. In addition, it is believed that all neurons or normal cells are naturally committed to die unless a survival factor such neurotrophin blocks this death through its receptors (Ichim et al., 2012a).

Conclusion

We investigated p75NTR pattern and protein expression in the *nax* mouse with severe cerebellar anomalies and Pc degeneration and in the WT sibling cerebellum. The expression of p75NTR is undistinguishable to the HSP25 expression pattern in the adult mouse cerebellum. In the *nax* cerebellum, the p75NTR pattern is similar to that of HSP25 and there is no significant difference between *nax* and WT siblings in p75NTR protein expression.

6.5 Limitations of the study

Preparing animal samples

The first difficulty we faced was preparing embryos from E9 to E18 for our first study (mesencephalic derived cerebellar nuclei neurons), the designated time by animal facility (2 pm) affected the precise time of breeding because of wideness of breeding time. To reduce this effect we changed the breeding time from 2 pm to 8 pm, but as a result it was our own responsibility to prepare mice for breeding each night.

For PAP/SNCA double ablation mice, the mice were a gift from Dr. Fiona Parkinson. After receiving first group of embryos and adult mice the colony was discontinued and we could perform no more studies.

In *Acp2* mutant mice, the viability of the mutant mice is very low.

Establishing embryonic culture and DiI administration

Dissociated cerebellar culture is well established in our lab, establishing embryonic culture was really time consuming to have optimum situation for mice embryos. DiI is a lipophilic dye when administrated to the mesencephalon there was high amount of DiI contamination. This dye was replaced by Fast DiI, which has the ability to become crystalized and form bars to easily administer to the mesencephalon. This dye is 50% faster than the normal DiI (move quicker). When we administer DiI/Fast DiI/hoechst and follow dye by time lapse we understand that these are not ideal for time laps imaging and finding better candidate dyes are suggested for future studies.Electroporation for embryos are not available in our institute to label cells with GFP and trace migrating cells using time laps imaging.

Otx2 immunohistochemistry method

Until now, no one was able to show Otx2 positive cells in cerebellar primordium by immunohistochemistry or in situ hybridization. The modulation of our standard operating procedure (SOP) was another difficulty to be optimized for Otx2 and it took around one year and half to optimize this antibody.

6.6 Future direction

Use of transgenic mice is a strong tool for proving many biological incidences. In the future, I plan to check these early cerebellar nuclei neurons in GFP- tagged *Otx2* mouse (green fluorescent protein). In collaboration with Dr. Thomas Lamonerie lab in Nice, France I already received GFP- tagged *Otx2* mouse embryos (E9.5, 10.5. 12.5 and 14.5) (Fossat et al., 2007). My plan is to co-label these cells with EGFP (enhancer green fluorescent protein) and SNCA, Otx2, p75NTR, Lmx1a and Tbr1. In another step to empower and provide more conclusive support of my hypothesis strongly by lineage tracing, I am in process of receiving another transgenic mice *Otx2*-CreER (*Otx2*$^{tm1.1(cre/ERT2)Mgoe}$/Cnrm). In this transgenic mice, the *Otx2* allele is replaced with CreER gene by homologous recombination and it is shown that by E 9.5, homozygous mutant embryos are characterized by the absence of forebrain and midbrain regions (Acampora et al., 1995, Millet et al., 1996). My plan is to conditionally silence this gene in different embryonic days by administering tamoxifen and evaluate the existence or trace Otx2 positive mesencephalic derived cerebellar nuclei cells in different intervals. When I follow the Otx2 positive cells between E9-P5, my findings will show if they are present in the cerebellar primordium. Briefly, I want to administer tamoxifen (Tx) for inducing recombination, the Otx2-CreERT2 mice will be treated with 0.1 mg/g body weight tamoxifen (Sigma) in order to induce

recombination. The injection will be at embryonic day (E) 9, E10, E11, E12 and E13. The termination will be as follow: E10 (Tx at E9), E11 (Tx at E9 and E10), E12 (Tx at E9, E10 and E11), E13 (Tx at E9, E10, E11 and E12), E14 (Tx at E9 , E10, E11, E12 and E13), E15 (Tx at E9 , E10, E11, E12 and E13), E16 (Tx at E9 , E10, E11, E12 and E13) and E18 (Tx at E9 , E10, E11, E12 and E13) and Postnatal day (P) 5 (Tx at E9 , E10, E11, E12 and E13).

When we follow the Otx2 positive cells between E9-E18, our findings showed that they are still present in the cerebellar primordium. In future, it is possible to follow these cells postnatally and check them for investigating any anomalies in ASD mice models or ataxia mice models.

We are only at the beginning of the understanding of the role of mesencephalic-derived cerebellar nuclei neurons that accompany the neuronal fibers at the core of the cerebellar primordium, and further studies are required to determine the precise molecular mechanism and functional significance of this putative signaling center.

CHAPTER SEVEN: REFERENCES

7.1 REFERENCES (CHAPTER ONE)

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CHAPTER EIGHT: GLOSSARY

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Embryonic stages in cerebellar afferent development

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Abstract

The cerebellum is important for motor control, cognition, and language processing. Afferent and efferent fibers are major components of cerebellar circuitry and impairment of these circuits causes severe cerebellar malfunction, such as ataxia. The cerebellum receives information from two major afferent types – climbing fibers and mossy fibers. In addition, a third set of afferents project to the cerebellum as neuromodulatory fibers. The spatiotemporal pattern of early cerebellar afferents that enter the developing embryonic cerebellum is not fully understood. In this review, we will discuss the cerebellar architecture and connectivity specifically related to afferents during development in different species. We will also consider the order of afferent fiber arrival into the developing cerebellum to establish neural connectivity.

Keywords: Mossy fiber, Climbing fiber, Neuromodulatory fiber, Cerebellar primordium

Introduction

The cerebellum is responsible for coordinating movement and maintaining equilibrium, and it is also involved in behavioral, cognitive and emotional functions [1–4]. Proper cerebellar function depends on well-organized neuronal connections and the integration of afferent and efferent fibers throughout cerebellar circuitry [5]. The cerebellum can be divided into two longitudinal regions along the rostral to caudal axia, which are anatomically named the vermis and hemisphere. Mediolaterally, each of these regions is folded into lobes, lobules and folia. Traditionally, the cerebellum is divided into 3 lobes that are further subdivided into 10 lobules. Lobules I-X numbering is used for the lobules of the vermis and the corresponding lobules of the hemisphere are indicated with the prefix H (e.g. [6–8]). Recent studies suggest that the fundamental architecture of the cerebellum is organized into five transverse zones based on gene expression and afferent fiber termination; the anterior zone (AZ: lobules I-V in mice), the central zone (CZ: lobules VI-VII, which is further subdivided into anterior (CZa) and posterior (CZp) components [9, 10]), the posterior zone (PZ: lobules VIII-dorsal lobule IX), and the nodular zone (NZ: ventral lobule IX and lobule X) (e.g. [11–18]). The boundaries of these zones do not align with the lobes and lobules, but do correspond to the pattern of afferent termination, and thus provides a more functionally relevant way of dividing the cerebellum.

The three-layered cerebellar cortex contains six main neuronal cell types: stellate and basket cells in the molecular layer, Purkinje cell somata in the Purkinje cell layer, and granule cells, Golgi cells, and unipolar brush cells in the granular layer. Neurons of the cerebellar nuclei are located close to the roof of the fourth ventricle deep within the cerebellar white matter. The cerebellar nuclei and the lateral vestibular nucleus constitute the sole output (efferent) of the cerebellum, and play a central role in cerebellar circuitry and function (e.g. [1, 19–22]).

The cerebellum is connected to the brainstem by three pairs of peduncles (superior, middle, and inferior), which are the pathways by which cerebellar afferents and efferents enter and exit [23, 24]. Cerebellar afferents can be grouped into two major types: mossy fibers and climbing fibers. Mossy fibers constitute the majority of afferent fibers in the adult cerebellum and arise from multiple

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sources. There are four main groups of mossy fibers that project to specific parts of the cerebellum: 1) the somatosensory projections (spinocerebellar and trigeminocerebellar fibers), which originate from the spinal cord and the trigeminal system (ganglion and nuclei) respectively, and project primarily to lobules I-VI and lobule VIII (e.g. [23, 25–27]); 2) vestibulocerebellar projections, which originate from the vestibular system (ganglion and nuclei) and terminate in ventral lobules IX and the flocculus with the adjacent ventral flocculonodular [28, 29]; 3) reticulocerebellar projections, which originate from the lateral reticular nuclei, the paramedian reticular nucleus and the nucleus of Roller, and nucleus reticularis tegmenti pontis terminate throughout the cerebellum, but generally mirror the projections of the spino/trigeminocerebellar and vestibulocerebellar systems (e.g. [30–33]) pontocerebellar projections, which originate from the pontine nuclei and terminate throughout the cerebellum, especially in the vermis and hemispheres of lobules VI-VII (central zone), but not in the nodulus and flocculus [34–36]. The nucleus prepositus hypoglosi is also a source of a mossy fiber system that projects restricted regions of crura I and II as well as the caudal vermis and vestibulocerebellum [37]. Mossy fiber afferents communicate with cerebellar nuclei neurons and with Purkinje cells through granule cells/parallel fibers [24, 38–40]. Climbing fibers are exclusively derived from the inferior olivary complex and enter the contralateral cerebellum through the inferior cerebellar peduncle [41, 42]. Climbing fibers directly synapse with the cerebellar nuclei and Purkinje cells (the sole output cells of the cerebellar cortex) [43–45]. In addition, a third set of afferents not categorized as mossy or climbing fibers exists, which terminate in all three layers of the cerebellar cortex and the cerebellar nuclei. Their functional role is neuromodulation and thus, they are categorized as neuromodulatory cerebellar afferents (i.e., serotonergic, noradrenergic, acetylcholinergic, dopaminergic, and histaminergic neuromodulatory afferents) (e.g. [1, 46–49]).

Extension and direction of early afferent fibers (axon pioneers) to their target neurons during development is controlled by genes and molecules, which mostly play a role in repulsion attraction (e.g. [50]). Several families of these proteins have been identified and among them four well-characterized families play an important role in axon guidance and development such as; Ephrin/Eph [50, 51], Semaphorins/ Neuropilins, [52, 53], Netrin [54, 55], and Robo/Slit [56, 57]. Molecules, such as integrins, fasciclin and neural cell adhesion molecules (NCAMs), are important in pioneer axon development by providing a substrate that promotes outgrowth of growth cones [58]. In addition, morphogens, initially characterized based on their effects on early patterning, are being increasingly implicated in axon guidance [50]. Morphogens found to be essential in cerebellar development include the Wnts family [59], Sonic hedgehog (Shh) [60, 61] and bone morphogenetic proteins (BMPs) [62]. These molecules from cell surfaces and intracellular signaling pathways provide cues that enable growing axons to terminate at their target cells. The Eph/Ephrin gene family has been indicated as a possible molecular pathway involved in regulating afferent patterning in both chick and mouse embryonic cerebella [63–65]. Engrailed-2 is important in development of cerebellum and regulates mossy fiber projections [66, 67]. UNC-5, a Caenorhabditis elegans protein that is essential for dorsal guidance of pioneer axons is important in development of the rostral cerebellum [68]. It has also been suggested that axonal development in the cerebellum is regulated by WNT-7a signaling [69]. Furthermore, gene mapping studies have shown that precerebellar nuclei including inferior olivary nuclei neurons likely originate from an area expressing Ptf1a, Oli3 and Wnt1 (e.g. [70]). Brn3 expression has been shown to be necessary for inferior olivary nuclei development [71]. Gene expression appears to be conserved across vertebrates as the inferior olivary nuclei of zebrafish also express ptf1a and brn3a [72]. It is known that the cerebellum and inferior olive express neurotrophins and their receptors that are involved in climbing fiber development [73]. It is also known that some genes are expressed differently in subsets of mossy fibers (e.g. somatostatin) and climbing fibers (e.g. corticotropin releasing factor) [74]. However, the regulatory roles of these genes and proteins in cerebellar afferent projections are poorly understood and will be interesting to investigate further to understand cerebellar circuit formation during cerebellar development.

The basic organization of afferent and efferent cerebellar connections appears to be conserved throughout evolution [75]. While there is much information regarding sources, pathways, terminations, and functions of afferents in the adult cerebellum, little attention has been paid to the subject during embryonic development. The order of afferent fiber arrival during cerebellar development is likely to be important in setting up the principal cerebellar circuits, but this is poorly understood. The basic spatiotemporal pattern of cerebellar afferents appears to be common between mammals, avians, reptiles, amphibians, and fish (e.g. [76, 77]). Therefore, in this review, we integrate information from different species in an attempt to resolve the order of afferent fiber arrival into the developing cerebellum.

Review

Development of cerebellar afferents

Mossy fibers

Mossy fiber projections probably play a critical role in establishing and organizing cerebellar circuitry during

early embryonic development. Conventionally, it is believed that mossy fibers enter the early embryonic cerebellum and target the Purkinje cells. However, it has been demonstrated that mossy fiber contacts with Purkinje cells are temporary and transient. Postnatally, as the granular layer matures, mossy fibers displace from Purkinje cells and synapse with their adult targets, the granule cell dendrites (e.g., [14, 77–81]). This multistage process of mossy fiber relay on timing and order of arrival during cerebellar development could be required for proper circuit formation and/or for afferents to pattern the target tissue.

Somatosensory pathway

Somatosensory input is carried into the cerebellum via spinocerebellar and trigeminocerebellar tracts.

Spinocerebellar afferents

The spinocerebellar afferent system is comprised of the dorsal spinocerebellar, ventral spinocerebellar, cuneocerebellar, rostral spinocerebellar tracts and from the central cervical nucleus which collectively convey proprioceptive information to the cerebellum. However, dorsal spinocerebellar tract component from lamina V and cuneocerebellar component from the internal cuneate nucleus transmits exteroceptive information to the cerebellum. The spinocerebellar afferents originate from diverse laminae of the spinal cord and can be classified into crossed and uncrossed tracts according to the cell origin and fiber course [82]. The cerebellum receives proprioceptive information from the hind limbs through the dorsal spinocerebellar tract (uncrossed) and the ventral spinocerebellar tract (crossed) [82, 83]. The dorsal spinocerebellar tract originates from the dorsal horn (mainly dorsal thoracic nucleus that is also known as Clarke's column) and provides a major mossy fiber input to the spinocerebellum, which plays a significant role in the control of posture and locomotion [84, 85]. The input conveyed by the ventral spinocerebellar tract originates from neurons of the dorsal and ventral horn and it monitors activity of spinal interneuronal networks [82, 86, 87]. The cuneocerebellar and rostral spinocerebellar tracts are the upper limb equivalents of the dorsal and ventral spinocerebellar tracts, respectively. The sources of these tracts are well known: the cuneocerebellar tract originates from the external and internal cuneate nucleus and the gracile nucleus in the medulla oblongata and the rostral spinocerebellar tract originates from the cervical spinal cord. The termination of all spino- and cuneocerebellar tracts is in bilateral, alternating symmetrically disposed longitudinal aggregates of mossy fiber terminals. The spinocerebellar afferents project to the cerebellum through the inferior cerebellar peduncle (non-crossed) and the superior cerebellar peduncle (crossed), and terminate in the anterior zone (lobules I-V) and posterior zone (lobules VIII-IXd) [28, 88].

Clinically, the spinocerebellar system is involved in a heterogeneous group of disorders classified collectively as spinocerebellar ataxia [89]. Spinocerebellar ataxia is a neurodevelopmental and neurodegenerative disease characterized by progressive incoordination of movement and degeneration of the cerebellar cortex and spinocerebellar pathways [89, 90]. It is believed that the cerebellar circuit organization, which is established during early cerebellar development, may be altered in ataxia (e.g. [91]).

Development of the spinocerebellar afferents

Spinocerebellar mossy fiber projections to the cerebellum during prenatal development have been studied in mice using *in vitro* tract tracing techniques (e.g. [22, 92]). Results of these studies have shown that at embryonic day 12/13 (E12/13), neuronal sources of spinocerebellar mossy fibers from the caudal cervical spinal cord are present, but there are no projections to the cerebellum. At approximately E13/14, spinocerebellar projected fibers have been shown to be present in the rostrolateral portions of the cerebellum adjacent to the isthmus region. Spinocerebellar fibers progress farther into the cerebellum and the number of crossed fibers increases as aging occurs [22]. In rats, tract-tracing methods have shown that spinocerebellar fibers project to the cerebellum in two successive groups: the first group is present in the cerebellum perinatally and the second group reaches the cerebellum by postnatal day 3 (P3) and targets the vermis of the anterior lobe and pyramis [93]. However, this timing was revised by Ashwell and Zhang, who showed that spinocerebellar fibers may be present in the developing rat cerebellum at around E15 [92, 94]. The external cuneate neurons are generated from the caudal rhombic lip between E13 and E15 in mice and may enter into the cerebellum during E13-E15 and thereafter [7, 95]. In the rat, cuneocerebellar fibers arrive in the developing cerebellum by E16/17 ipsilaterally via the inferior cerebellar peduncle [6].

In the chicken, spinocerebellar projections have been studied by injecting WGA-HRP into the spinal cord of embryos at different stages of development. These studies showed that spinocerebellar fibers entered the cerebellum at around the 7th incubation day [96]. In the African clawed frog, *Xenopus laevis,* spinocerebellar afferents were studied using HRP tracer techniques. At approximately stage 50, before the formation of the limbs, the ventral spinocerebellar projections appeared to be present in the cerebellum prior to entry of the dorsal spinocerebellar tract [97]. Although primary spinocerebellar projections from spinal ganglion cells to the cerebellum have been demonstrated in the adult frog [98, 99], they were not detectable during development [97]. Tract tracing methods

have also been used to study spinocerebellar projections in reptiles including turtles (*Pseudemys scripta elegans* and *Testudo hermanni*), lizards (*Varanus exanthematicus*), and snakes (*Python regius*). The basic pattern of cerebellar afferent projections appears to resemble that of other vertebrates. However, it has been suggested that there is no column of Clarke or central cervical nucleus in the reptilian spinal cord [99]. To our knowledge, no developmental studies have investigated spinocerebellar projections in reptiles. In the lesser spotted dogfish (*Scyliorhinus*), spinocerebellar afferents were shown to project to the developing cerebellum at around stage 32 (equivalent to around E14.5 in the mouse) [100, 101].

In summary, spinocerebellar fibers invade the developing cerebellum at around E13/14 in the mouse (e.g. [22, 92]), E15 in the rat [94], HH33 (Hamburger-Hamilton stages 33) in the chicken (around the 7th incubation day; [96]), stage 50 in *Xenopus laevis* [97], and at stage 32 in the dogfish [101, 102] (Tables 1 and 2).

Trigeminocerebellar afferents

The trigeminal system (trigeminal nerves, ganglion, and nuclei) is the largest cranial nerve, which has both sensory and motor components [103]. Peripherally, the trigeminal nerve is comprised of three branches: ophthalmic (V1, sensory), maxillary (V2, sensory), and mandibular (V3, sensory and motor), which converge together on the trigeminal ganglion. From the trigeminal ganglion, a single large sensory root enters the brain stem at the level of the pons and terminates at the complex trigeminal nuclei, which consist of the spinal trigeminal nucleus, main (principal) trigeminal nucleus, and mesencephalic trigeminal nucleus. The motor component is derived from the basal plate of the embryonic pons, while the sensory component originates from the alar plate and the neural crest (e.g. [104]).

The trigeminal nerve carries temperature, pain and tactile information from the skin, jaws and teeth. In addition, it carries proprioceptive input primarily from the muscles of mastication to the mesencephalic trigeminal nucleus, which projects to higher centers to coordinate jaw movement in activities such as suckling, mastication, biting and speech (e.g. [105]). In adult rats, the mesencephalic and interpolaris trigeminal nuclei have direct projections to the cerebellum [106, 107]. There are also primary trigeminal afferent projections from the ganglion (probably from the mandibular branch) to the cerebellum of adult rats [108]. The primary trigeminocerebellar projections (originating from the trigeminal ganglion) run through the superior peduncle, whereas secondary trigeminocerebellar projections (originating from the trigeminal nuclei) run through the inferior peduncle, and both terminate in the same target areas in the cerebellum [26, 109].

In the cerebellum, the mesencephalic tract nucleus projects to the anterior lobe, the simple lobule (HVI), lobules VI, VIII, and the dorsal paraflocculus (e.g. mice [27], rats [109], and sheep [110]). The ventral group of the main (principal) and spinal tract nuclei project to all lobules in the vermis and hemispheres, whereas the dorsal parts of these nuclei have a more restricted projection field including the vermis of the lobules VI, VII, and IX and corresponding hemispheres [110]. In the rat, the trigeminal nucleus oralis projects to ipsilateral orofacial portions of four major tactile areas (crura I and II, the paramedian lobule, and the uvula) of the cerebellar cortex [111, 112]. Retrograde tracing has shown that most of the spinal trigeminal nuclei project to the paramedian lobule in the tree shrew (*Tupaia glis*), but the principal and mesencephalic trigeminal nuclei do not [113]. Trigeminocerebellar afferents have been demonstrated in *Xenopus laevis* [114]. In mallards, the trigeminocerebellar afferents terminate predominantly in lobules V, VI and VII, and possibly in

Table 1 Timing of cerebellar afferent fiber arrival in different species during the embryonic period

Afferent fiber	Mouse	Rat	Chicken	Xenopus	Reptile	Fish[a]
Spinocerebellar a	E13/14	E15	HH33/E7	Stage 50	n/a	Stage 32
Trigeminocerebellar a	n/a	E22/P0	n/a	Stage 48	n/a	Stage 32
Vestibulocerebellar a	E11-13	13-14/15	n/a	Stage 48	n/a	n/a
Reticulocerebellar a	E13/14	E16/17	n/a	Stage 48	n/a	Stage 32
Pontocerebellar a	E16	perinatal	n/a	n/a	n/a	n/a
Olivocerebellar a	E14/15	E17	E9	n/a	n/a	Stage 32
Serotonergic cerebellar a	postnatal	postnatal	n/a	Stage 56	n/a	n/a
Noadrenergic cerebellar a	E14	E17	n/a	n/a	n/a	n/a
Cholinergic cerebellar a	n/a	n/a	n/a	n/a	n/a	n/a
Dopaminergic cerebellar a	n/a	n/a	n/a	n/a	n/a	n/a
Histaminergic cerebellar a	n/a	n/a	n/a	n/a	n/a	n/a

Note: a, afferent fiber

The "n/a" means that there is no data available for afferents projection to the cerebellum during embryonic development in these species

[a]This is the only stage that has been studied; therefore everything arrives at stage 32

Table 2 Timing of cerebellar afferent fiber arrival in different species during embryonic and postnatal stages

Afferent fiber	Embryonic									postnatal	
Days	2	4	6	8	10	12	14	16	18	0/2	adult
Spinocerebellar a				▶			▶	▶	▶		
Trigeminocerebellar a				▶						▶	
Vestibulocerebellar a				▶		▶	▶				
Reticulocerebellar a				▶			▶	▶			
Pontocerebellar a								▶		▶	
Olivocerebellar a					▶		▶		▶		
Serotonergic cerebellar a										▶ ▶	▶
Noradrenergic cerebellar a							▶		▶		
Cholinergic cerebellar a											
Dopaminergic cerebellar a											
Histaminergic cerebellar a											

a, afferent fiber
▶mice; ▶rat; ▶chicken; ▶reptile?; ▶xenopus; ▶fish?

lobule IV, and are ipsilateral except for the vermal area [115]. The secondary trigeminocerebellar projections have been demonstrated in mammals (e.g. [116]), birds [117], reptiles [100], and amphibians [114].

Development of the trigeminocerebellar afferents

In the central nervous system, mesencephalic neural crest cells emerge and start migrating at the 4- to 7-somite stage in mice, some of which give rise to the primary sensory neurons that form the mesencephalic trigeminal nucleus [104]. At E8.5, by the 10-somite stage, the first group of neurons can be identified in the rostral part of the mesencephalon [104]. This is the earliest neuronal development that has been detected in the central nervous system; this is in contrast to previous information, which indicated that neurons first differentiated in the mouse central nervous system by E9-E10 [104]. It has been demonstrated that the cells of the mesencephalic trigeminal nucleus are born before E11 in rats [118], and as early as E8 in mice [104]. Although formation of this nucleus takes place during early neural tube development, Ashwell and Zhang have suggested that the trigeminocerebellar afferents project to the cerebellum around E22 in rat [94].

Information regarding the development of trigeminal projections in the avian cerebellum is limited. In reptiles, the presence of trigeminocerebellar projections has been demonstrated [100], but not studied during development. In amphibians (*Xenopus laevis*), the secondary trigeminocerebellar projection (from the descending nucleus of the trigeminal nerve) has been shown in the cerebellum at approximately stage 48 [97]. In addition, trigeminocerebellar fibers can be observed in the cerebellum at stage 32 in the dogfish [102] (Tables 1 and 2). Thus, the trigeminal system components seem to be among the earliest neurons and fibers to develop in the vicinity of the cerebellar primordium. However, the lack of clarity in the timing of the developing trigeminocerebellar projections requires further research.

Vestibulocerebellar afferents

The vestibulocerebellar system, which is important for maintaining balance and equilibrium, provides sensory input to the cerebellum [119]. The vestibular nucleus includes four subnuclei: superior, inferior, medial, and lateral [119]. The lateral vestibular nucleus does not receive vestibular root fibers, but Purkinje cell axons from the B zone (the lateral part of the vermis), therefore, it is a cerebellar rather than a vestibular nucleus [28]. Projections from the vestibular system to the cerebellum are comprised of two components: primary vestibular mossy fiber afferents (i.e. originate from the vestibular ganglion) and secondary vestibular mossy fiber afferents (i.e. originate from the vestibular nuclei) [120]. Vestibular nerve projections to the cerebellum are primarily limited to the nodulus, uvula, and flocculus [120, 121]. Additionally, in bottom deep fissures (basal part of the lobules), lobules I and II, where they are distributed in parallel longitudinal aggregates [122], the flocculus does not receive primary vestibular mossy fibers [123]. The "vestibular commissure" of Larsell would form the basis of the flocculonodular lobe [124]. Primary and secondary vestibulocerebellar afferents have been shown in amphibians [125, 126], reptiles [100, 127], birds [128] and mammals (e.g. [116, 129–131]).

Development of vestibulocerebellar afferents

It is suggested that vestibular ganglion neurons are born on E10-E14 in mice [131], and E11-E13 in rats [132]. Vestibular nuclei projection neurons are born in

the neuroepithelium lining the fourth ventricle between E12 and E14 in the rat in the following order: the lateral (approximately E12), superior (approximately E13), inferior (approximately E13-14), and medial vestibular nucleus (approximately E13-14) [132, 133]. Historically, the classical observation of Tello and Ramon Y Cajal (1909) suggested that vestibular fibers were the first afferents to reach the cerebellum at six days prenatal in the mouse. However, the timing was slightly revised some years later, with vestibular axons from the vestibular ganglia being the first afferents to enter the cerebellum at E13-14/15 in the rat and E11-12/13 in mice [94, 133, 134]. From E15, collateral branches from primary vestibulocerebellar afferents form contacts with the developing vestibular nuclei within the subventricular zone [134].

Vestibular nuclei are generated at around E2 in chickens [135]. However, to date there are no data for avian vestibulocerebellar fiber development. Developmental studies are currently lacking on reptilian vestibulocerebellar projections, but in amphibians (*Xenopus laevis*), it has been demonstrated that vestibulocerebellar projections, which mainly arise bilaterally in the nucleus vestibularis caudalis, appear at around stage 48 [97] (Tables 1 and 2). Although the vestibulocerebellar projections are suggested to be the earliest afferents to invade the cerebellar primordium, further studies using a greater range of experimental approaches are required to confirm this important and fundamental process.

Reticulocerebellar afferents

A mixture of neurons and nerve fibers located in the brainstem make up a poorly defined group of nuclei collectively referred to as the reticular formation. The reticular formation is a primitive neuronal network upon which a more morphologically organized mass of neurons have developed during evolution [135, 136]. The reticular formation can be divided into three bilateral longitudinal columns based on both structure and function: median (raphe nuclei), medial, and lateral nuclei [136]. The lateral reticular nucleus can be further divided into three subnuclei (parvocellular, magnocellular and subtrigeminal) that project to the cerebellum. The caudal and ventral part of the lateral reticular nucleus projects bilaterally, mainly to the vermis, its rostral and dorsal part, that receives the "ipsilateral forelimb tract" and a collateral projection of the rubrospinal tract projects to the ipsilateral hemisphere [137]. The paramedian pontine reticular nucleus projects bilaterally to the cerebellar lobules VI, VII and the crura I and II that symmetry about the medline [32]. The parvocellular portion and adjacent magnocellular portion project primarily to the vermis, the remaining magnocellular subnucleus projects to the hemispheres, and the subtrigeminal subnucleus projects to the flocculonodular lobe. Neurons of the reticular formation project to the cerebellar cortex and cerebellar nuclei through the superior/middle/inferior cerebellar peduncles [138]. Reticular formation projections, which are aligned with the cerebellar architecture, carry out functions such as orofacial behaviour, including eye blink reflexes [39, 138].

Development of reticulocerebellar afferents

In the rat, the reticulocerebellar nucleus is not identifiable until birth when the axons of these neurons reach the cerebellum, even though reticular nucleus neurons are born on E12/13 [30]. Other studies have suggested that axons from the lateral reticular nuclei begin arriving in the developing cerebellum by E16/17 in rat (equivalent to E13/14 in mouse; [7]) via the inferior cerebellar peduncle [139]. At present, detail regarding the reticulocerebellar projections in the avian cerebellum is lacking (e.g. [96]). In reptiles, projections to the cerebellum were demonstrated from the reticular formation [100], but developmental studies in this system are currently required. Reticulocerebellar connections have been demonstrated experimentally at stage 48 in *Xenopus laevis*, [97]. In the dogfish, reticulocerebellar neurons are observed at stage 32 from the reticular formation of the mesencephalon and rhombencephalon [101, 102] (Tables 1 and 2).

The reticular formation is a complicated organization of nuclei that is involved in both sensory and motor pathways, and is generally one of the earliest nuclei to grow ontogenetically and phylogenetically. It seems that the reticulocerebellar projections are established early in development as a fundamental requirement to establish the basic functional circuitry of this pathway.

Pontocerebellar afferents

The pontine nuclei, located at the basilar portions of the pons (basilar pontine nuclei), receive most of their input from the cerebral cortex [140]. The majority of mossy fibres innervating the hemispheres originate from the pontine nuclei [138]. All pontocerebellar fibers end as mossy fibers in the cerebellar nuclei and cortex [141, 142]. The nucleus reticularis tegmenti pontis project to the cerebellar nuclei (pronounced in lateral and posterior interposed nuclei), the paraflocculus and crus II of the ansiform lobule [143]. The size of the pontine nuclei increases in parallel with the size of the associated cerebral cortex, reaching peak development in humans [136].

Development of pontocerebellar afferents

Pontine neurons arise from the lower rhombic lip around E14 to E17 and tangentially migrate to the ventral pons in the rat. At E16.5, migrating pontine neurons are present in the pontine nuclei with their axons laterally projecting towards the developing cerebellum [95]. These axons cross the midline and enter the cerebellum through the

middle peduncle [144]. The neurons of the pontine nucleus are generated from the rhombic lip at E16 in mice [145], while in the rat, pontocerebellar nuclei are first labelled at birth [92]. It has been suggested that pontine mossy fibers enter the cerebellum perinatally in rat [92, 146]. The pontocerebellar projections are found in birds and mammals [33, 128, 145–148], but projections from the primordial pontine nuclei to the cerebellum in reptiles and amphibians have not been demonstrated [100] (Tables 1 and 2).

Climbing fibers

Climbing fibers are the second major type of cerebellar afferents. Unlike mossy fibers, which originate from numerous sites in the nervous system, climbing fibers originate exclusively from the inferior olivary nucleus (e.g. [41, 149–151]). The inferior olivary nucleus can be subdivided into three major subnuclei: the principal olivary nucleus, which projects mainly to the cerebellar hemisphere, the dorsal accessory olivary nucleus, which projects to the vermis and hemispheres, and the medial accessory olivary nucleus, which projects to the vermis and paravermis in the rat [152]. Climbing fibers relay information to the cerebellum from several regions, such as the cerebral cortex, thalamus, red nucleus, reticular formation, trigeminal nuclei, and spinal cord. It is evident that inferior olivary neurons are necessary for proper cerebellar function, possibly in motor learning [153] and in motor timing [154]. The inferior olivary nucleus is a common feature of amphibians, reptiles, birds and mammals. Inferior olivary nucleus projections have been demonstrated experimentally in the frog [155, 156], and termination of these afferents in a longitudinal pattern has been demonstrated in the avian cerebellum [157]. The olivocerebellar circuit is essential for normal cerebellar function [158].

Development of olivocerebellar afferents

Several studies support that inferior olivary neurons originate from the caudal region of the rhombic lip, r6-8 (reviewed in [70]). Gene mapping studies have shown that IO neurons likely originate from an area expressing Ptf1a, Oli3 and low levels of Wnt1 (reviewed in [70, 159]. Brn3 expression has been shown to be necessary for ION development [71]. Post-mitotic inferior olivary cells in the caudal hindbrain migrate circumferentially toward the ventral floor plate of the medulla where the mature inferior olivary nuclei will form [139, 160]. Inferior olivary neurons originate at E10-11 in mice [70], E12-14 in rats [160], and E3-5 in chicks [161].

Axons of inferior olivary neurons begin migration before the cell bodies, such that the axons reach the cerebellum around the same time that the cell bodies settle in the ventral floor plate [162]. Organization of the inferior olivary subnuclei is clear by E10 in chicks [159] and E18/19 in rats [42, 139]. Olivocerebellar axons cross the midline and enter the contralateral cerebellum through the inferior cerebellar peduncle [42]. These axons reach the cerebellum around E14/15 in mice [163], E17 in rats [7, 164], and E9 in chicks [165]. Within the cerebellum, inferior olivary axons give off thick and thin branches. The thick branches form the climbing fibers, which terminate in the cerebellar cortex and synapse directly with Purkinje cells [42]. Immature climbing fibers have been shown to occur in the Purkinje cell region as early as P0 in mice [9, 166], and synapses have been found to occur as early as E19 in rats [167]. Early in postnatal development, each Purkinje cell is innervated by multiple climbing fibers. In rats, this occurs as early as P3 and peaks at P5, with an average of 3.5 climbing fibers per Purkinje cell [168, 169]. Climbing fiber innervation is subsequently reduced leaving a single strong excitatory synapse [163]. Mono-innervation occurs by P15 in rats [169]. In mice, this process is longer, ending around the third postnatal week, with defined early and late stages [151, 170]. The thin branches of the inferior olivary neurons terminate on the cerebellar nuclei [171]. In the North American opossum (*Didelphis marsupialis virginiana*), olivary axons reach the developing cerebellum at approximately postnatal day 4 [172].

From an evolutionary perspective, the lamprey, an ancient jawless fish/agnathan, possesses a region comparable to the cerebellum [173]. The presence of the inferior olivary nuclei in agnathans has not been demonstrated; however, the presence of the inferior olivary nuclei has been demonstrated in jawed fishes/gnathostomes (e.g. teleosts [72] and chondrichthyans [101, 102]). The inferior olivary nuclei in fish are considered to be homologous to the medial accessory olivary nucleus of mammals [174]. In the dogfish (chondrichthyan), the olivocerebellar projections develop after spinocerebellar projections [101, 102], similar to birds and mammals. These projections are first obvious during intermediate stage 32, when the first climbing fibers reach the cortex [101, 102]. In dogfish, Purkinje cells have matured by stage 32 [175], unlike mammalian development in which climbing fibers enter the cerebellum before Purkinje cells have matured. In zebrafish (teleost), the inferior olivary nucleus can be distinguished at 4 days post-fertilization in the ventromedial region of the posterior hindbrain, and climbing fibers have been shown to innervate Purkinje cells at 5 days post fertilization [72]. The development and organization of the olivocerebellar system appears to be conserved throughout vertebrates (e.g. [76, 175]). Overall, the arrival of inferior olivary projections to the cerebellum appears to happen at roughly equivalent developmental stages in fish, birds, and rodents (Tables 1 and 2).

Neuromodulatory afferents

In addition to mossy fibers and climbing fibers, other cerebellar afferent pathways have also been discovered. These afferents originate from subnuclei of the reticular formation and are traditionally called neuromodulatory cerebellar afferents. They project to the cerebellum through the superior, middle, and inferior peduncles and in general terminate in all three layers of the cerebellar cortex. The neuromodulatory cerebellar afferents influence cerebellar circuitry in various ways and can be categorized into 5 groups based on the chemical messengers used for communication: serotonergic, noradrenergic, acetylcholinergic, dopaminergic, and histaminergic neuromodulatory afferents (e.g. [46–49]). In addition to their largely unknown function, the distribution and development of these afferents projection to the cerebellum is poorly understood.

Cerebellar serotonergic afferents

The serotonergic fibers are the largest modulatory afferent fibers to the cerebellum [49]. A combination of retrograde transport studies and serotonin immunohistochemistry has shown that serotonergic cerebellar fibers arise from a few of the reticular formation subnuclei: the nucleus reticularis gigantocellularis, the nucleus reticularis paragigantocelularis, and also from the pontine reticular formation in the nucleus reticularis pontis oralis [176]. In rats, serotoninergic fibers project to all parts of the cerebellar cortex. However, the distribution of fibers across the cerebellum is not uniform, with different densities projecting to the granule cell and molecular layer and to different lobules. The fibers also terminate in the Purkinje cell layer. The distribution is found to be thickest in lobules VIII-X of the vermis. A dense uniform plexus of serotonergic cerebellar fiber projections is present in the cerebellar nuclei of the cat cerebellum [49, 176, 177]. It seems that serotonergic fibers have modulatory effects on Purkinje cells and cerebellar nuclei [178, 179].

From a clinical point of view, the serotonergic abnormalities in the cerebellum may be involved in patients with developmental neuropsychiatric disorders such as autism and attention deficit/hyperactivity disorder (ADHD) [180, 181]. Serotonin may disturb behavioral responses that are associated with cerebellar timing signals, such as the eye-blink reflex [49, 182].

Development of cerebellar serotonergic afferents

The expression of the serotonergic phenotype begins at E12 just caudal to the mesencephalic flexure, in rostral raphe nuclei [183, 184]. It is suggested that serotonergic projections to the cerebellum develop during the postnatal period [185], which coincides with the perinatal development of the cerebellar cortex. It has been shown that serotonergic fibers are present in the chicken [186], the opossum [172], and at stage 56 in the cerebellum of Xenopus (which may have originated from inferior raphe nucleus) [187] (Tables 1 and 2).

Cerebellar noradrenergic afferents

Noradrenergic afferents are the second largest modulatory input to the cerebellum [46] and originate from the locus coeruleus [188], a system that supplies noradrenaline (norepinephrine) throughout the central nervous system. The locus coeruleus is located in the pons, which is involved in physiological functions such as arousal, sleep/wake cycles, nociception, anxiety, stress, learning and memory [189, 190]. Noradrenergic fibers project to all parts of the cerebellar cortex and are found around Purkinje cell dendrites, glomeruli, granule cell dendrites, and cerebellar nuclei [116]. The significance of noradrenergic cerebellar projections is suggested to be correlated with learning [191].

Development of cerebellar noradrenergic afferents

It is suggested that the locus coeruleus neurons are among the first central nervous system neurons to arise during ontogeny. In mice, these neurons originate from dorsal r1 between E10.5 to E12.5, and their axons may reach the cerebellum by E14 [192]. They establish axonal connections to their target areas while migrating into their nuclear area in 4th ventricle at around E17 in the rat (Tables 1 and 2). Noradrenergic axons are demonstrable in the developing cerebellum for the first time at E17 and the peak of innervation is reached at P10 [193]. This hyper-innervation is transitory and declines to adult values at around P20 [193].

Cerebellar cholinergic afferents

Cholinergic input to the cerebellum is thinly dispersed, creating a diffuse plexus of beaded fibers in the cerebellar cortex and cerebellar nuclei [194–196]. It has been proved that a subset of vestibular projections to the cerebellar cortex are cholinergic [196]. However, the reticular formation is the principal source of cholinergic cerebellar afferents [48]. In the rat cerebellum, cholinergic fibers originate in the pedunculopontine tegmental nucleus, the lateral paragigantocellular nucleus, and, to a lesser extent in various raphe nuclei [48]. Cholinergic afferents terminate in the granule cell layer and molecular layer of the cerebellum in rat, rabbit, and cat [194]. The cholinergic fibers in the cerebellar nuclei form a moderately dense network, and could, in principle, have a significant effect on neuronal activity [48].

Development of cerebellar cholinergic afferents

Using specific binding sites for [^{3}H]quinuclidinyl benzylate, Mallol et al. suggested that cholinergic fibers are

present at a low level in the cerebellum at birth [197]. However, there is limited information regarding cholinergic cerebellar afferents during development.

Cerebellar dopaminergic afferents

There are sparse dopaminergic projections to the cerebellar cortex and nuclei, which appear to originate mostly from the ventral tegmental area [198]. It has been demonstrated that most dopamine receptor proteins are present in Purkinje cells [199]. Dopamine can influence plasticity in Purkinje cells [46].

Development of cerebellar dopaminergic afferents

Dopaminergic neurons are born at approximately E10-E14 in mice (E12-E16 in rats) from the ventral midbrain/mesencephalon floor plate [200]. However, there is no on the development of dopaminergic cerebellar afferent projections.

Cerebellar histaminergic afferents

Histamine plays a role as a neurotransmitter or a neuromodulator in the cerebellum. Interestingly, there are direct fiber connections from the tuberomammillary nucleus of the hypothalamus to the cerebellum [201], suggesting that histamine is involved in signal transmission from the hypothalamus to the cerebellum [202]. It has been proposed that histaminergic fiber projections to the cerebellum are important in regulating the level of behavioral arousal, and in the control of autonomic and emotional functions [46, 199]. Histaminergic cerebellar afferents have been shown to be present in the cerebellum of the rat, guinea pig and human [203–205].

Development of cerebellar histaminergic afferents

The first histamine-immunoreactive neurons have been demonstrated at E13 in the border of the mesencephalon and metencephalon [206]. However, additional information is needed regarding further development of histaminergic cerebellar afferents.

Conclusions

The order in which cerebellar afferent projections appear throughout the development of embryonic mice and rats, in comparison with the other species, has shown that there is a general pattern in the arrival of cerebellar afferents during embryonic development. This suggests that the appearance of early cerebellar afferents takes place during a short period; those from the vestibular nuclei, inferior olive, trigeminal, and reticular nuclei arrive first, followed by the connections from spinal cord. Knowing the order in which cerebellar afferents arrive will provide information about the role that afferents may play during early embryonic development of the cerebellar primordium. The earliest afferent projections to the cerebellum may be involved in development, acting as a scaffold for subsequent incoming afferents, thereby establishing cerebellar circuitry and function. Limited knowledge about the order of cerebellar afferent development during the embryonic period prevents appropriate conclusions from being made, and requires further experimentation in this area. There is no developmental information available regarding some cerebellar afferents, particularly in the neuromodulatory category. Thus, further research is required to close this knowledge gap. In addition, because less sensitive methods were used in the past we suggest that the study of cerebellar afferent fiber development should be revisited by utilizing genetically encoded tools to detect the sequence of afferent projections during developing.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MRB and HM participated in preparing mossy fiber section, KB, MB, and HM participated in climbing fiber preparation, PA, MB and HM contributed in preparing neuromodulatory afferents, and BY and HM are coordinated in editing the draft of the manuscript, designed by HM. All authors read and approved the final manuscript.

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The Embryology and Anatomy of the Cerebellum

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Abstract The cerebellum is an important structure in the central nervous system that controls and regulates motor and non-motor functions. It is located beneath the occipital lobe and dorsal to the brainstem. Today, we know much about its complex circuitry and physiology. The cerebellum has a well-defined and highly organized structure. The cortex of the cerebellum contains eight neuronal cell types and receives input from a variety of sites within the CNS and processes the information in a uniform manner. The cerebellum projects to a variety of different sites within the CNS to regulate motor function. Although much has been discovered regarding the complex architecture of the cerebellum, there are significant gaps in our understanding of the broader role of the cerebellum in brain function. In this chapter, we will review briefly the embryological development of the cerebellum and provide an overview of the anatomy of the cerebellum.

Keywords Cerebellum • Embryology • Anatomy • Histology • Function

Introduction

The cerebellum (latin: 'little brain') is located in the posterior cranial fossa and is involved in the regulation of posture, motor coordination, balance, and motor learning. More recently, it has been proposed that it also plays a role in emotion and cognition. The cerebellum consists of a midline region referred to as the vermis, a narrow paravermal area immediately adjacent to the vermis, and large hemispheres on either side. Well-defined fissures divide the cerebellum in a rostral caudal direction into an anterior lobe, posterior lobe, and flocculonodular lobe. The anterior and posterior lobes are divided further, into lobules and folia (in human), which greatly increases the surface area of the cerebellum. The cerebellum consists of a uniform

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layer of cortical grey matter overlying white matter that surrounds four pairs of cerebellar nuclei (CN). The cerebellar cortex consists of three layers: molecular layer, Purkinje cell layer, and granule cell layer. The molecular layer is the outermost layer and is largely a synaptic layer, containing the connections of a number of neurons (e.g., basket and stellate cells) with the dense dendritic arborizations of the Purkinje cells, whose cell bodies are the predominant component of the Purkinje cell layer. The innermost layer of the cortex is the granule cell layer containing Golgi cells, Lugaro cells, unipolar brush cells, and the highly abundant granule cells. Almost all of the neurons of the cerebellar cortex use either the excitatory neurotransmitter glutamate or the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Glutamate is used by the granule cells and unipolar brush cells while the remainder of the cortical neurons use GABA. The CN are primarily composed of large projection neurons that use glutamate as a neurotransmitter and project to nuclei of the thalamus and brainstem. These neurons represent the principal output of the cerebellum. A smaller number of CN neurons are GABA-ergic and project to the inferior olivary nucleus of the medulla. The cerebellum is considered an outstanding model in the research of neurogenesis and circuit assembly because of its well organized structure.

Embryology of the Cerebellum

During prenatal development of the nervous system, the central nervous system originates from the area of the ectoderm known as the neural plate. The neural plate thickens as a result of cell proliferation and then begins to invaginate and thus forms the neural groove. The invagination of the neural groove continues until the lateral edges of the neural groove (neural fold) fuse to form the neural tube through a process referred to as neurulation. As the edges of the neural groove fuse to form the neural tube, which detaches from the ectoderm, a population of the neuroectodermal cells dissociate from the neural fold as the neural crest cells [1]. The rostral extent of the neural tube develops into the prosencephalon, mesencephalon, and rhombencephalon. The prosencephalon undergoes further development to form the telencephalon and diencephalon. The mesencephalon does not undergo further division while the rhombencephalon divides into the metencephalon and myelencephalon. Caudal to the rhombencephalon, the neural tube develops into the spinal cord. The cerebellum develops from the dorsal portions (i.e., the alar plate) of the metencephalon and the neural folds, the latter referred to as the rhombic lips. The alar plate of the rostral metencephalon undergoes bilateral expansion in the dorsolateral region to form the rhombomere 1 (r1). These rostral extensions of alar plate eventually join in the midline to form the vermis of the cerebellum. As the cerebellum begins to form, initially from the dorsal r1, it rotates 90° before fusing at the midline as the vermis [2]. This rotation of dorsal r1 results in the conversion of rostral–caudal axis seen in the early neural tube, into the medial–lateral axis seen in the mature cerebellum (the wing-like bilateral cerebellar primordia) [3]. As the bilateral

cerebellar primordia fuse, the midline vermis is derived from the rostro-medial ends while the cerebellar hemispheres are derived from the more caudo-lateral components of the rhombencephalon [4].

The neurons that reside within the cerebellum are derived from two distinct germinal zones: the ventricular zone and the rhombic lip. The ventricular zone is the neuroepithelium of the alar plate of the rhombencephalon that eventually forms the roof of the fourth ventricle. The neurons derived from the ventricular zone include the Purkinje cells, candelabrum cells, Golgi cells, Lugaro cells, stellate cells, and basket cells. All of these neurons use GABA as a neurotransmitter and reside in the outer two layers of the three layered cortex, except for the Golgi and Lugaro cells of the granular layer [5, 6]. The neurons derived from the rhombic lip use glutamate as a neurotransmitter. This includes the large neurons of the CN (projecting to the diencephalon and brainstem), unipolar brush cells, and granule cells, the most numerous cell in the brain.

Anatomy and Histology of the Human Cerebellum

Functional Divisions of the Cerebellum

The cerebellum is a highly organized structure that is attached to all three components of the brainstem (the midbrain, pons, and medulla) [7]. Fissures divide the cerebellum into three lobes in the rostro-caudal plane. The primary fissure, seen on the superior surface of the cerebellum, separates the anterior lobe from the posterior lobe, while the posterolateral fissure, seen on the inferior surface of the cerebellum, separates the large posterior lobe from the narrow and much smaller flocculonodular lobe. The flocculonodular lobe consists of bilateral extensions of cerebellar cortex called flocculi that are connected to the inferior portion of the vermis called the nodulus. During development, once the anterior and posterior lobes form, smaller lobules begin to form. The lobules undergo further infolding which leads to the formation of folia, which are particularly prominent in human cerebellum. The structure of the folia is consistent throughout the cerebellum, with a three-layered cortex overlying the white matter consisting of the axons projecting to and from the cortex (Fig. 1).

The cerebellum is organized into three functional divisions based on their connections to other brain sites and their respective roles in regulating movement and other non-motor functions. The phylogenetically oldest component of the cerebellum is the flocculonodular lobe. The cortex of this lobe receives input from the vestibular apparatus on the ipsilateral side as well as input from the vestibular nuclei of the brainstem. Therefore, the flocculonodular lobe is commonly referred to as the vestibulocerebellum. The connections of the vestibulocerebellar cortex to the vestibular nuclei are reciprocal, and the cortex of the vestibulocerebellum is the only component of the cerebellar cortex that sends projections directly to sites outside the cerebellum (i.e., the vestibular nuclei of the brainstem) [7]. The vestibulocerebellum participates in the control of balance and eye movements.

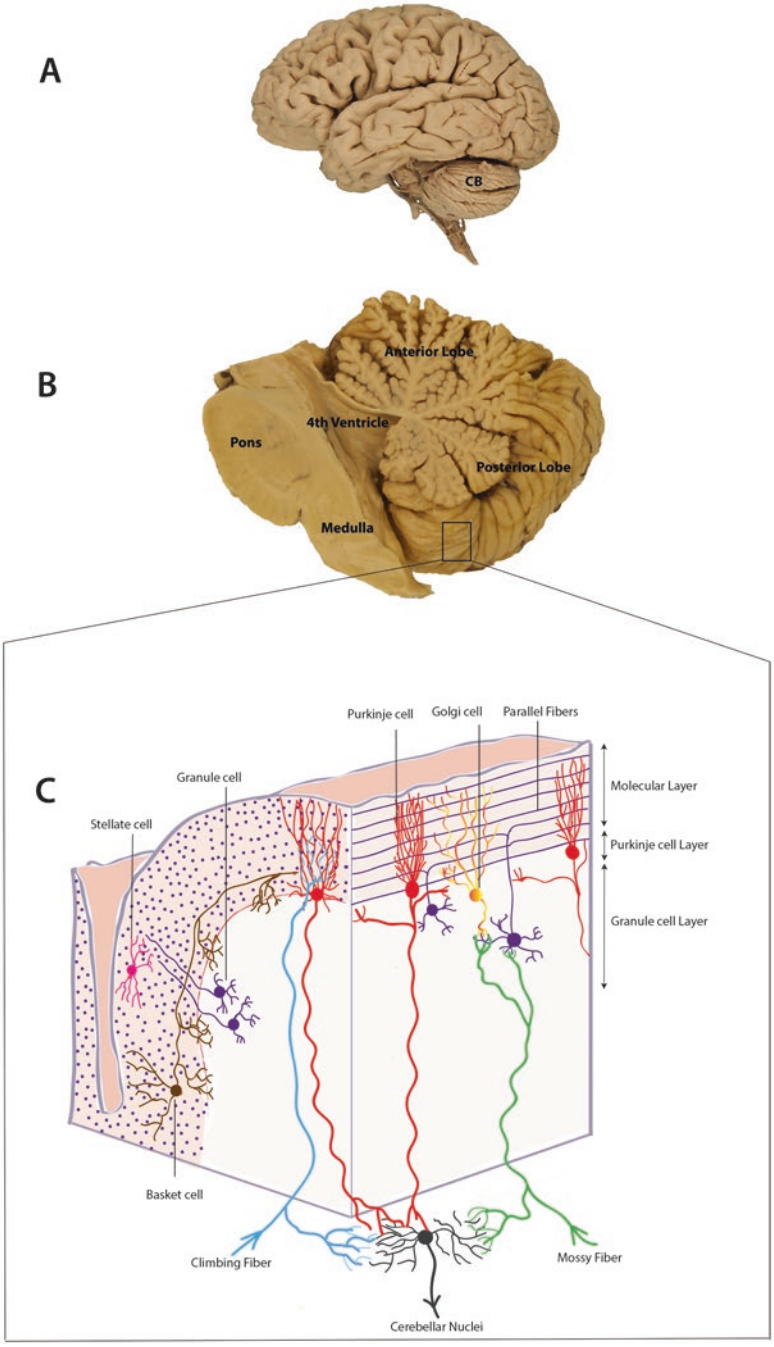

Fig. 1 (**A**) Location of the cerebellum in situ. (**B**) Hemisected view of the cerebellum showing the vermis, the locations of the anterior and posterior lobes, and its anatomical relationship to the brainstem. (**C**) Schematic representation of the cerebellum showing the mossy fibre and climbing fibre inputs to the cerebellar cortex. The mossy fibres contact the granule cells and send collaterals to the cerebellar nuclei while the climbing fibres make contact with the dendrites of the Purkinje cells and may also send projections to the cerebellar nuclei. The granule cells project to the molecular layer and bifurcate to form the parallel fibres that contact the Purkinje cell dendrites as well as the basket cells and stellate cells. The Golgi cells receive input from mossy fibres and also project into the molecular layer of the cortex

The second functional component of the cerebellum consists of the midline vermis of the anterior lobe and a narrow portion of cortex on either side of the vermis referred to as the paravermal cortex. This component is referred to as the spinocerebellum as the bulk of the input to the spinocerebellum is provided by ascending tracts in the spinal cord. The spinocerebellum receives input from the dorsal spinocerebellar tract that transmits proprioceptive, cutaneous, and pressure information from the lower extremity (on the ipsilateral side) [7]. It also receives input from the cuneocerebellar tract, which carries somatosensory information from the upper extremity.

A third major input into the spinocerebellum is the ventral spinocerebellar tract. It transmits information regarding the activity of circuits within the spinal cord involved in regulating motor activity. Additionally, the spinocerebellum also receives inputs from a number of brainstem nuclei including the reticular formation. The spinocerebellum participates in regulating axial and proximal limb muscle musculature involved in balance, posture, and locomotion.

The third and largest functional component of the cerebellum is the pontocerebellum (also referred to as the cerebrocerebellum). It is also the phylogenetically newest component of the cerebellum. It consists of the large hemispheres immediately lateral to the spinocerebellum and receives input principally from the contralateral cerebrum, via the pons. Descending corticopontine fibres from widespread areas of the cerebral cortex (particularly frontal and parietal lobes) project to pontine nuclei of the basilar pons [7]. The neurons of these nuclei send their projections across the midline to project to the cortex of the pontocerebellum. The pontocerebellum is particularly well developed in higher mammals and participates in regulating the coordination of the distal limb musculature as well as playing a role in motor learning.

Cerebellar Cortex

The cortex of the cerebellum is remarkable in its uniformity and segregates into three layers: the outer molecular layer, the Purkinje cell layer, and the inner granule cell layer [6]. The molecular layer contains stellate cells and basket cells but is dominated by the dendrites and axons of other neurons. The molecular layer receives input from neurons of the inferior olivary nucleus of the medulla, and these fibres are referred to as climbing fibres. The climbing fibres make abundant excitatory synaptic connections with the proximal dendritic tree of Purkinje cells [8]. The molecular layer also receives abundant excitatory input from the granule cells of the cerebellar cortex. Granule cells send their axonal projections to the molecular layer cortex where the axons bifurcate and form parallel fibres that run parallel to the cortical surface and make synaptic connections with the dendritic tree of numerous Purkinje cells. The stellate cells of the molecular layer are inhibitory interneurons that use GABA as a neurotransmitter, and these cells are located primarily in the outer part of the molecular layer. These cells also receive input from parallel fibres

and make synaptic contacts with the dendritic tree of Purkinje cells. Finally, the basket cells of the molecular layer also use GABA as a neurotransmitter and are located in the inner portion of the molecular layer. Basket cells receive excitatory input from the parallel fibres of the granule cells and make abundant inhibitory connections on the cell bodies of Purkinje cells in a basket-like manner.

The Purkinje cell layer consists of the large cell bodies of the Purkinje cells, which send an extensive dendritic tree into the molecular layer, and candelabrum cells. The dendritic tree of a single Purkinje cell receives excitatory inputs from a single climbing fibre of the inferior olivary nucleus and numerous inputs from parallel fibres of the granule cells. The Purkinje cell is of particular importance because it represents the sole output of the cerebellar cortex. It uses GABA as a transmitter and projects almost solely to the CN. The exception to this rule is the Purkinje cells of the vestibulocerebellum that also project to the vestibular nuclei of the brainstem. Interspersed between the Purkinje cells within this layer are candelabrum cells that are also GABA-ergic neurons that send their dendritic projections into the molecular layer. The functional significance of these cells is poorly understood.

The granule cell layer is the innermost layer of the cortex and consists of granule cells, Golgi cells, unipolar brush cells, and Lugaro cells. The granule cells are the most abundant neuron in the human nervous system and are packed tightly within the granule cell layer. They receive excitatory input from mossy fibres, which are the principal input into the cerebellum. Mossy fibres originate from numerous sites within the nervous system, including pontine nuclei, nuclei of the reticular formation, vestibular nuclei, and the fibres of the spinocerebellar tracts of the spinal cord. The granule cells, which use glutamate as a neurotransmitter, extend their axons into the molecular layer where they bifurcate into the aforementioned parallel fibres and connect with the dendritic tree of up to hundreds of Purkinje cells. The activity of the granule cells plays a critical role in determining the activity of the Purkinje cells. Additionally, the parallel fibres of the granule cells also shape the activity of other cell types of the cerebellar cortex, including Golgi, stellate, and basket cells. The Golgi cells are relatively large cells that are more abundant in the superficial portion of the granule cell layer, nearer to the Purkinje cell layer [9]. These are also GABA-ergic neurons and extend their dendrites into the molecular layer where they receive synaptic input from the parallel fibres of the granule cells. The Golgi cells also make synaptic connections to the granule cell dendrites, thereby providing a source of inhibition to the granule cell. Unipolar brush cells are neurons within the superficial part of the granule cell layer and like granule cells use glutamate as a neurotransmitter. These cells are more abundant in the vestibulocerebellum than other parts of the cerebellum and are closely associated with mossy fibres and project to granule cells and other unipolar brush cells. The final cell intrinsic to the cerebellar cortex is the Lugaro cell. These are GABA-ergic neurons found primarily in the superficial portion of the granule cell layer. Their dendrites may extend into the molecular layer, while their axon is restricted to the granule cell layer where they make connections with Golgi cells.

Within the cerebellar cortex, the connections and links between the parallel fibres of granule cells and the dendrites of inhibitory cells such as Purkinje cells and

others, and the connections between the mossy fibres and Purkinje cells (and other neurons), make a unique and uniform microcircuitry observed with great consistency in all parts of the cerebellar cortex.

Cerebellar Nuclei (CN)

There are four pairs of CN embedded within the white matter of the cerebellum (dentate, emboliform, globose, and fastigial) that receive input from the cerebellar cortex as well as the collaterals of fibres projecting to the cerebellar cortex [10]. The first cerebellar neurons generated are neurons of the CN. These cells originate from the rhombic lip and migrate tangentially to the nuclear transitory zone (NTZ). The CN constitute the sole output of the cerebellum (excepting some of the Purkinje cells of the vestibulocerebellum), and they receive the output of the cerebellar cortex from the inhibitory Purkinje cells. In addition to the inhibitory inputs from the Purkinje cells, the CN receive the collateral excitatory inputs from mossy fibres and climbing fibres projecting to the cortex. The majority of CN neurons are excitatory neurons that project to sites outside the cerebellum, including the thalamus, red nucleus, reticular formation, and vestibular nuclei. However, a small population of CN neurons are GABA-ergic, and these neurons project to the inferior olivary nucleus.

The fastigial nucleus is the smallest and most medial of the CN. The neurons of this nucleus receive input from the Purkinje cells of the vestibulocerebellum (i.e., flocculonodular lobe). In addition, the fastigial nucleus also receives input from Purkinje cells of the vermis that receive input from the vestibular apparatus either directly or indirectly via the vestibular nuclei. The neurons of the fastigial nucleus project to the brainstem vestibular and reticular nuclei. As mentioned previously, some of the Purkinje cells of the flocculonodular lobe also send direct (inhibitory) projections to brainstem vestibular nuclei.

Lateral to the fastigial nuclei are the globose and emboliform nuclei, also referred to collectively as the interposed nuclei. These nuclei receive input from the Purkinje cells of the vermis and paravermal areas of the anterior lobe of the cerebellum, which in turn receives input from the cuneate nucleus (via the cuneocerebellar tract) and the accessory cuneate nucleus and Clarke's nuclei (via the dorsal spinocerebellar tract). The interposed nuclei send projections primarily to the red nucleus of the midbrain and the ventrolateral nucleus of the thalamus. The latter nucleus relays this information to the primary motor, supplementary motor, and pre-motor cortices of the frontal lobe.

The dentate nucleus is the largest and most lateral of the CN. It receives inhibitory input from the Purkinje neurons of the large lateral hemispheres and excitatory input from the collaterals of the climbing fibres and mossy fibres projecting to the lateral hemispheres that have their origin in the inferior olive and basilar pontine nuclei, respectively. The neurons of the dentate nucleus project to the red nucleus and the ventrolateral nucleus of the thalamus, which relays the information to the motor cortices of the frontal lobe.

Cerebellar Peduncles

The cerebellum connects to the midbrain, pons, and medulla via three peduncles: the superior, middle, and inferior peduncles, respectively [7, 11]. The superior cerebellar peduncle consists primarily of efferent fibres from the dentate and interposed nuclei projecting to the contralateral red nucleus and ventral lateral nucleus of the thalamus. The cerebellar efferents of the spinocerebellum that project to nuclei of the reticular formation also pass through this peduncle. The cerebellar afferents contained within this peduncle are primarily the fibres of the ventral spinocerebellar tract that project as mossy fibres to the granular layer of the spinocerebellum and send collateral branches to the interposed nuclei.

The middle cerebellar peduncle is a massive bundle of afferent fibres connecting nuclei in the basilar pons to the contralateral cerebellar cortex. These fibres project as the mossy fibres to the granular layer of the large lateral hemispheres and send collateral branches to the dentate nucleus.

The inferior cerebellar peduncle contains fibres connecting the cerebellum to the medulla and consists of the restiform body and the juxtarestiform body. The juxtarestiform body primarily consists of the reciprocal connections of the cerebellum and the vestibular nuclei. The afferent fibres within the juxtarestiform body form the mossy fibres projecting to the granular layer of the vestibulocerebellum. The efferent fibres of the juxtarestiform body include Purkinje cell axons of the vestibulocerebellum and the projections of the fastigial nucleus to vestibular and reticular nuclei of the brainstem. The restiform body contains fibres projecting from the brainstem and spinal cord to widespread areas of the cerebellum. This includes fibres of the dorsal spinocerebellar tract and cuneocerebellar tract projecting to the spinocerebellar cortex as mossy fibres with collateral projections to the interposed nuclei. In addition, fibres originating in the inferior olivary nucleus projecting to the molecular layer of the cerebellar cortex as climbing fibres (with collateral projections to the dentate nucleus) are also contained within the restiform body. The inferior olivary nucleus receives inputs from spinal, vestibular, cranial, and cortical descending signals. The neurons of the inferior olivary nucleus relay somatosensory and noxious stimuli. A single climbing fibre of the inferior olivary nucleus projects to a few Purkinje cells, while each Purkinje cell makes synaptic connections with only one climbing fibre.

The cerebellar cortex also receives projections from a variety of areas of the brain including the locus coeruleus (noradrenergic fibres), raphe nuclei (serotonergic fibres), mesencephalic tegmentum (dopaminergic fibres), and the hypothalamus (histaminergic fibres) [7, 11]. These inputs to the cerebellum terminate in all three layers of the cerebellar cortex as well as the CN. These projections to the cerebellum are commonly referred to as neuromodulatory cerebellar afferents and are thought to decrease the activity of Purkinje cells. The precise distribution and development of these afferent projections to the cerebellum is not well understood. Further research is required to better understand their role in cerebellar function.

Cerebellar Function

The function of the cerebellum can be broadly divided into three categories as set by the three functional divisions described above.

The vestibulocerebellum consists of the midline nodulus and the bilateral flocculе [7, 11]. The mossy fibres projecting to the cortex originate in the vestibular ganglion of the vestibular apparatus and the vestibular nuclei of the brainstem. The Purkinje cells of the cortex send inhibitory projections to the fastigial nucleus as well as the ipsilateral vestibular nuclei. The fastigial nucleus, which serves as the principal cerebellar nucleus of the vestibulocerebellum, sends excitatory bilateral projections to the vestibular nuclei. These connections to the vestibular nuclei pass through the inferior cerebellar peduncle. These projections play an important role coordinating the vestibular ocular reflex via the ascending vestibular nuclei projections contained within the medial longitudinal fasciculus to control eye movement in response to vestibular feedback. The vestibular nuclei also send fibres descending the spinal cord as the vestibulospinal tract. These fibres play a critical role in maintaining balance through activation of the anti-gravity muscles of the lower body. The fastigial nucleus also sends ascending projections via the superior cerebellar peduncle to the ventrolateral nucleus of the contralateral thalamus. This information is subsequently relayed to the corticospinal neurons of the anterior corticospinal tract (medial motor system) involved in maintaining posture and balance through activation of the axial musculature. Lesions of the vestibulocerebellum are often characterized by nystagmus and vertigo, resulting from dysregulation of the connections between vestibular nuclei and brainstem nuclei regulating eye movement. Lesions of the fastigial nucleus are also commonly associated a wide-based gait as a result of instability or ataxia of the axial musculature.

The spinocerebellum consists of the midline vermis and paravermal areas of the cerebellum [8]. The mossy fibres projecting to the cortex are largely the fibres of the spinocerebellar and cuneocerebellar tracts. To a lesser extent, the spinocerebellum also receives input from reticular, vestibular, and pontine nuclei. Although the interposed nuclei receive the bulk of the collateral fibres derived from the ascending inputs into the spinocerebellar cortex, the fastigial nucleus also receives some these collaterals. Similarly, the Purkinje cell axons of the paravermal areas of the spinocerebellar cortex project primarily to the interposed nuclei while the vermal areas project to the fastigial nucleus. The fibres projecting from the interposed nuclei exit the cerebellum via the superior cerebellar peduncle. The majority of these fibres project to the ventral lateral nucleus of the contralateral thalamus, and this information is relayed to supplementary motor, pre-motor, and primary motor cortex involved in regulating the limb musculature. The descending projections of these cortical areas will primarily form the lateral corticospinal tract (i.e., the lateral motor system). The interposed nuclei also send projections to the red nucleus and the reticular nuclei to effect changes in the descending rubrospinal and reticulospinal fibres involved in regulating the activity of the spinal cord motor neurons projecting to the upper and lower limbs. The vermal areas of the spinocerebellum that

project to the fastigial nucleus are primarily involved in regulating axial musculature. As described above, the fastigial nucleus projects to the reticular and vestibular nuclei of the brainstem and the ventrolateral nucleus of the thalamus. Lesions of the vermal portion of the spinocerebellum are characterized by axial muscle instability, while lesions of the paravermal portions of the spinocerebellum produce ataxia affecting the upper and lower limbs.

The pontocerebellum consists of the lateral hemispheres of the cerebellum and constitutes the largest of the three functional components of the cerebellum. The mossy fibres projecting to the pontocerebellar cortex are almost entirely crossing pontocerebellar fibres. These fibres originate in the pontine nuclei of the contralateral basilar pons and enter the cerebellum via the middle cerebellar peduncle. These fibres send collaterals exclusively to the dentate nucleus, which also receives input from the Purkinje fibres of the pontocerebellum. The fibres of the dentate nucleus exit the cerebellum through the superior cerebellar peduncle. These fibres cross the midline within the tegmentum of the caudal midbrain and continue rostrally where some fibres enter into the red nucleus. The neurons of the red nucleus project to the inferior olivary nucleus of the medulla, which projects back to the pontocerebellum and dentate nucleus forming a feedback loop to the cerebellum. The majority of the fibres originating from the dentate nucleus continue past the red nucleus to the thalamus. The fibres terminate on neurons in the ventrolateral nucleus and to a lesser extent in the ventroanterior nucleus of the thalamus. The thalamic neurons contacted by the neurons of the dentate nucleus project rostrally to a large portion of the motor cortices, with an emphasis on the primary motor cortex. The descending neurons from the primary motor cortex form a large component of the lateral motor system. These projections play a critical role in coordinating the muscle activation required for performing fine motor skills of the distal extremities, particularly of the upper limb. The cortical areas regulated by the thalamic relays of the dentate nucleus also play an important role in the planning of motor activity. Lesions of the pontocerebellum are characterized by a decreased ability to control the distance, velocity, and power of movement performed by the extremities. Lesions of the pontocerebellum are commonly characterized by intention tremor and difficulty in performing rapid alternating movements of the hand (e.g., pronation and supination). The latter deficit is referred to as dysdiadochokinesia. These deficits underscore the importance of the pontocerebellum in regulating fine motor skills.

Blood Supply of the Cerebellum

The cerebellum is supplied with arterial blood via three cerebellar arteries: the posterior inferior cerebellar artery (PICA), the anterior inferior cerebellar artery (AICA), and the superior cerebellar artery (SCA) [12]. These arteries are derived from the vertebral–basilar arterial system that supplies the posterior circulation of the brain. The bilateral vertebral arteries pass through the foramen magnum and shortly after entering the cranium, the PICA branches off the vertebral artery. The

PICA supplies the cortex of the posterior portion of the inferior cerebellum and the inferior portion of the underlying white matter. It also supplies the fibres of the inferior cerebellar peduncle. The vertebral arteries fuse in the midline, near the junction of the pons, and the medulla, to form the basilar artery, and the AICA branches off the basilar artery immediately anterior to this junction. The AICA supplies the cortex of the anterior portion of the inferior cerebellum and the underlying white matter. Distal branches of the AICA may extend into the lateral portion of the dentate nucleus. The AICA also supplies the posterior part of the middle cerebellar peduncle, while circumferential branches of the basilar artery supply the anterior portion of the middle cerebellar peduncle. The most lateral edge of the inferior surface of the cerebellum is generally the watershed area of the PICA and the AICA.

The SCA attaches to the basilar artery immediately posterior to the bifurcation of the basilar artery into the paired posterior cerebral arteries. The SCA supplies the superior surface of the cerebellum and the bulk of the white matter of the cerebellum. It also supplies the CN except for the lateral portion of the dentate nucleus that may be supplied by the AICA. The SCA also supplies the superior cerebellar peduncle together with branches of the posterior cerebral artery.

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REVIEW
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Neuronal Migration During Development of the Cerebellum

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Neuronal migration is a fundamental process in central nervous system (CNS) development. The assembly of functioning neuronal circuits relies on neuronal migration occurring in the appropriate spatio-temporal pattern. A defect in the neuronal migration may result in a neurological disorder. The cerebellum, as a part of the CNS, plays a pivotal role in motor coordination and non-motor functions such as emotion, cognition and language. The excitatory and inhibitory neurons within the cerebellum originate from different distinct germinal zones and migrate through complex routes to assemble in a well-defined neuronal organization in the cerebellar cortex and nuclei. In this review article, the neuronal migration modes and pathways from germinal zones to the final position in the cerebellar cortex and nuclei will be described. The cellular and molecular mechanisms involved in cerebellar neuronal migration during development will also be reviewed. Finally, some diseases and animal models associated with defects in neuronal migration will be presented.

Keywords: neuron, migration, cerebellum, development, mechanism

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INTRODUCTION

The structural and functional development of the central nervous system (CNS) depends on neurogenesis, neuronal migration and circuit formation. This is a complex sequence of events involving a variety of molecular pathways. Neuronal migration is an essential phenomenon for normal development as it brings cells into appropriate spatial relationships with other cells

Abbreviations: Acp2, lysosomal acid phosphatase 2; ApoER2, apolipoprotein E receptor 2; Astn, astrotactin 1; Atoh1, atonal homolog 1; BDNF, brain-derived neurotrophic factor; CAMs, cell adhesion molecules; CN, cerebellar nuclei; CNN, cerebellar nuclei neuron; CNS, central nervous system; CXCL12, chemokine ligand 12; CXCR4, chemokine receptor 4; Dab1, disabled-1; DCC, deleted in colorectal cancer; DCX, doublecortin; E, embryonic day; EAAT1, excitatory amino acid transporter; EGF, epidermal growth factor; ErbB4, Erb-B2 receptor tyrosine kinase 4; FoxP2, forkhead box protein P2; GABA, gamma amino butyric acid; GC, granule cell; GCP, granule cell precursor; GFAP, glial fibrillary acidic protein; GLASTs, glutamate receptors and transporters; HGF/SF, hepatocyte growth factor/scatter factor; i, isthmus; IN, interneuron; IZ, intermediate zone; KCC2, potassium-chloride co-transporter; LIS1, lissencephaly-1 homolog; Lmx1a, LIM homeobox transcription factor 1 alpha; m, mesencephalon; MAP, microtubule-associated protein; MDM2, mouse double minute 2 homolog; MZ, marginal zone; *Nf1*, neurofibromatosis type 1; NRG1, neuregulin-1; NTZ, nuclear transitory zone; P, postnatal day; Pax2, paired homeobox gene 2; Pax6, paired homeobox gene 6; PC, purkinje cell; PCC, purkinje cell cluster; PCP, purkinje cell plate; Pex2, peroxisomal biogenesis factor 2; Ptf1a, pancreas specific transcription factor 1a; RL, rhombic lip; Rp58, 58 KDa repressor protein; SDF-1, stromal derived factor 1; Sema3A, semaphorin 3a; Sema6A, semaphorin 6A; Shh, sonic hedgehog; SmoA2, smoothened A2; SVZ, subventricular zone; Tbr1, T-box, brain, 1; Tbr2, T-box, brain, 2; UBC, unipolar brush cells; uPAR, urokinase-type plasminogen activator receptor; VLDLR, very-low-density lipoprotein receptor; VPS18, vacuole protein sorting 18; VZ, ventricular zone.

(Marín et al., 2010). During development, newborn neurons form within the neuroepithelium, a proliferative layer of the neural tube. Under tightly controlled conditions, the newborn neurons migrate from their germinal zone and disperse throughout the CNS to reach their final destination where they subsequently become part of an appropriate lamination and neuronal circuit (Cooper, 2013). Cell polarity is required for neuronal migration which is dependent on cytoskeletal changes in concert with cell adhesion receptor systems that are regulated by a wide variety of molecules (Govek et al., 2011). Neuronal locomotion can be divided to three phases: (1) extension of the leading process; (2) nucleokinesis; and (3) retraction of the trailing process (Tsai and Gleeson, 2005). The main mode of neuronal migration is somal translocation which uses extracellular matrix components (Nadarajah and Parnavelas, 2002), glial fibers (Hatten, 1990), blood vessels (Tsai et al., 2016), axons (Takei et al., 2000) and possibly pia mater (Komuro and Yacubova, 2003) as substrates. Neuronal migration follows either a radial or a tangential migratory pathway, depending on the area of the developing nervous system in which the neurons originate. In radial migration, nascent neurons follow a track that is perpendicular to the neuroepithelial surface and the neurons proceed alongside radial glial fibers (Marín and Rubenstein, 2001). In contrast, the tangential migration of neurons is parallel to the pial surface (Nadarajah et al., 2001). There is also a dual phase neuronal migration referred to as a switching migration, which is a combination of tangential and radial migration (Kawaji et al., 2004).

In this article, the neuronal migration modes and the direction and pathways from origin to the final position during CNS development will be reviewed. Then, we will review the migration of cerebellar neurons with an emphasis on Purkinje cells (PCs). Finally, we will describe some diseases and animal models associated with defects in neuronal migration.

THE CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN NEURONAL MIGRATION

During neuronal migration, neuronal precursor cells move forward, switch their direction, or change their migration mode to reach their final position, which is fundamental for nervous system function. All of these processes are under an elaborate control system and have been studied extensively (**Table 1**). In this section, we will describe the main molecular and cellular mechanisms involved in neuronal migration during CNS development and then the migration of each cerebellar neuronal type will be described.

Migrating neurons exhibit highly polarized cell morphology in the direction of their movement. The polarized neurons are defined as having a leading process and a trailing process. The leading process is a structure that is similar to the growth cones of growing axons, whereas the trailing process is a short process at the posterior part of the cell. The formation of these processes is regulated by precise cellular and molecular mechanisms through which extrinsic and intrinsic signaling pathways change the cytoskeleton resulting in pulling and pushing forces (Matsuki et al., 2013; Nguyen and Hippenmeyer, 2013). The major structures that define the leading edge activity of migrating neurons are lamellipodia and filopodia (Kurosaka and Kashina, 2008). Initially a lamellipodium-like network forms and then filopodia form through the addition of monomers to filaments and assembly with adjacent filaments (Davies, 2013). Lamellipodia are broad membrane protrusions at the leading edge of cells that arise as a result of actin polymerization. Lamellipodia are dynamic structures that include protrusion and retraction activities (Krause and Gautreau, 2014). On the other hand, filopodia are thin protrusions of the lamellipodium plasma-membrane. The formation of filopodia is a highly dynamic process and these structures function as antennae to navigate and direct cell migration. The initiation and elongation of filopodia depends on the precise regulation of polymerization, crosslinking and assembly by various actin-associated proteins (Mattila and Lappalainen, 2008).

The movements of neurons are controlled by the generation, maintenance and remodeling of a leading process. The leading process of the neuron marks the direction of neuronal migration, followed by movement of the cell somata (somal translocation) along with the translocation of the nucleus (nucleokinesis), and finally the migrating neuron eliminates its trailing process. Leading processes interact with the surrounding microenvironment to guide neuronal movements (Nguyen and Hippenmeyer, 2013). The remodeling of the leading process will repeatedly initiate new migratory cycles until it reaches its final destination (Nguyen and Hippenmeyer, 2013). Cytoskeletal proteins such as microtubules, actin and actomyosin play important roles in nucleokinesis and cell locomotion. The centrosome is the main microtubule organizing center and as it moves forward, it pulls forward the longitudinal array of microtubules in association with the Golgi apparatus, which is followed by the movement of the nucleus. The absence of microtubules at the trailing part of the cell may initiate

TABLE 1 | Methods to study neuronal migration (Mannan et al., 2004; Baubet et al., 2012; Rahimi-Balaei et al., 2016).

Techniques for progenitor differentiation and neuronal migration	
Traditional	a) Analysis of fixed tissue
	b) *in vitro*: culture of individual cells
Advanced	a) Electron microscopy
	b) Autoradiography
	c) Tissue culture methods
	d) Chimeras
	e) X-ray crystallography
	f) Genetic labeling
Recent	a) Live imaging techniques
	b) Genetic labeling of distinct cell types in developing brain
	c) *In vitro* migration assay using dissociated neuronal cells migration (boyden chamber assays and gap closure assays)
	d) Real-time neuronal migration in embryonic brain slice assay (fluorescent dyes or XFP transgenes, lipophilic or vital dyes, such as DiI, DiO, CMTMR, Oregon Green plus dye- or transgene-coated gold particles *in utero* or *ex vivo* electroporation)
	e) Neuronal migration in embryonic brain explants in 3-D matrigel
	f) Embryonic culture
	g) Dynamic *in-silico* model for neuronal migration

contractions dependent on myosin II, and this pushing force on the nucleus results in moving forward and breaks adhesions at the trailing part of the cell. The role of actomyosin contraction at the back part of the cell also plays an important role in the migration of cortical interneurons (INs; Martini and Valdeolmillos, 2010). The somal translocation process is the main mode of neuronal migration during the early stage of embryonic development and includes the radially migrating neurons such as cerebellar granule cells (GCs) that move along the Bergmann glia fibers. A wide range of cellular events, including cell adhesion, modulate this migration (Hatten, 1999; Nadarajah et al., 2001; Sanada et al., 2004).

It has been shown that Lissencephaly-1 homolog, (LIS1, a member of the microtubule-associated proteins, MAPs) and doublecortin (DCX, a member of MAP that directly polymerizes purified tubulin into microtubules) are important in the translocation of the neuronal cell body during neuronal migration. Both molecules are components of an evolutionarily conserved pathway regulating microtubule function and cell migration (Gleeson et al., 1999; Feng and Walsh, 2001). In addition, the microtubule bundling that is accompanied by the action of dynein mediates coupling of the nucleus to the centrosome (modulating and stabilizing microtubules; Tanaka et al., 2004). In another study, it has been shown that LIS1 and dynein play a role in radial neuronal migration (Wynshaw-Boris and Gambello, 2001). In males, DCX mutations produce lissencephaly phenotypes similar to those associated with *LIS1* mutations (Gleeson et al., 1998). Recently, c-Jun N-terminal signaling pathway has gained attention as one of the critical regulators of neuronal mobility. Indeed, components of this pathway activate some specific brain proteins (e.g., by phosphorylation of the MAP1B and MAP2) which affect the stability of microtubules in neurons and neuronal migration (Tsai et al., 2016). In cerebellar GC migration, that is assisted by Bergmann glia, the majority of F-actin and myosin II is located at the front of the nucleus rather than the trailing end, suggesting these proteins may pull the soma forward (Solecki et al., 2009).

In addition to intrinsic factors discussed above, there are several extrinsic factors (also known as motogens) involved in neuronal migration. Gamma amino butyric acid (GABA) secreted by neurons themselves, acts as an extrinsic factor and accelerates their migration. In mice deficient in GABA, the migration rate of neurons is decreased, which is consistent with a role for GABA as a motogen (Inada et al., 2011). Hepatocyte growth factor/scatter factor (HGF/SF) is another extrinsic factor involved in migration. In mice lacking the urokinase-type plasminogen activator receptor (uPAR, a key component of HGF/SF activation), neurons exhibit abnormal migration from the ganglionic eminence, which leads to a reduced number of neurons in the frontal and parietal cortices (Powell et al., 2001).

The rate of cerebellar GC migration is controlled positively through the frequency of the intracellular calcium fluctuation and negatively regulates the rate of the extension of axonal growth cones. In cortical migratory interneurons, their motility is stimulated by the activation of GABA and glutamate receptors. An up-regulation of the potassium-chloride co-transporter (KCC2) plays a key role in reducing interneuron motility through its ability to reduce membrane potential upon gamma-aminobutyric acid A($GABA_A$) receptor activation, and decrease the frequency of intracellular calcium transients. Subsequently, during early postnatal weeks the expression of KCC2 is increased and early-born interneurons express higher levels of KCC2 compared to late-born interneurons (Bortone and Polleux, 2009).

The control of the specific direction of the migration in cortical neurons originating from the subventricular zone (SVZ) is a combination of the leading process and the use of scaffolds (such as the radial glia; Marín et al., 2010). However, there are additional factors such as semaphorin 3A (Sema3A), which acts like an attractant and is expressed in descending gradients across cortical layers, that guide newborn cortical neurons to the upper cortical layers (Chen et al., 2008). During development of pontine nuclei in the hindbrain, neurons reach the midline and Netrin-1 acts as a midline attractant and these neurons themselves express deleted in colorectal cancer (DCC), a Netrin-1 receptor), to assist these neurons to reach the midline (Yee et al., 1999). Neuregulin-1 (NRG1) is a member of the NRG family of proteins that contains an epidermal growth factor (EGF)-like motif that activates EGF receptor. It is expressed in the developing cortex and acts as a chemoattractant. Erb-B2 Receptor Tyrosine Kinase 4 (ErbB4), the NRG1 receptor, is expressed in migrating interneurons (Flames et al., 2004).

It is clear that several intrinsic and extrinsic factors are involved in the regulating neuronal migration. The mode of the neuronal migration, its direction, and finally the positioning of the neurons, which is important for neuronal circuit formation and function, are regulated by a complex molecular pathway that is currently not fully understood and need to be addressed in future. In the next section, we discuss direction of neuronal migration. The orientation and directionality of cell migration can be classified into two basic axes (radial and tangential) that use different types of substrates such as glial processes or neuronal axons (Rakic, 1990).

Radial Migration

Radial migration occurs in two opposite directions: (1) pial-directed migration in which neurons migrate toward the pial surface (or the outer neural tube surface); and (2) radially inward migration in which neurons migrate away from the pial surface. In the pial-directed radial migration, the neural progenitors or neuroepithelial derived cells migrate from their site of origin toward the pial surface to reach the mantle zone. Radial glial cells, which express glial fibrillary acidic protein (GFAP), play an important role in promoting the generation of neuronal progenitors and providing the migratory substrate during the neuronal migration (Tabata and Nakajima, 2003). Although pyramidal cells of the cerebral cortex are the classical example of pial-directed radial migration, a recent study has demonstrated this movement is not as straight forward as previously thought. Recently, it has been described that neurons may switch the mode of their migratory pathway (in the intermediate and subventricular zone, IZ/SVZ) before starting radial migration (Tabata and Nakajima, 2003).

During inward radial migration, neurons move away from the pial layer after their tangential migration. The typical examples for this mode of migration are cerebellar GCs and pontine nuclei neurons (Kawauchi et al., 2006).

Tangential Migration

Tangential migration occurs in two different manners: (1) directed; and (2) non-directed. In the directed manner of migration, many neurons and interneurons migrate tangentially from their site of origin toward a specific direction. This includes cerebellar granule cell precursors (GCPs), interneurons of the cerebral cortex, neurons of the pontine nuclei, Cajal Retzius cells, neurons of the lateral reticular nucleus, and neuronal migration from the telencephalon to the olfactory bulb. The interneurons of the cerebral cortex migrate between the pallium and subpallium and the neurons of the pontine nuclei migrate between rhombomeres (Nóbrega-Pereira and Marin, 2009). Cajal-Retzius cells play an important role during neuronal migration as they secrete Reelin to guide the radial migration of the projection neurons of the neocortex. These cells originate from the discrete pallium and by tangential migration they will colonize the surface of the entire cortex (Bielle et al., 2005; Gil-Sanz et al., 2013).

The non-directed manner of tangential migration is more complicated than other modes of the migration, in that some groups of neurons exhibit migrations in all directions of the tangential plane. For example, interneurons of the marginal zone (MZ) of the cerebral cortex migrate tangentially in different directions, or change their direction repeatedly, which is referred to as random walk (Tanaka et al., 2009).

Switching Migration (Mode and Direction)

Although many neurons migrate simply to reach their final destinations, for some neurons the migration is more complicated and involves a type of migration referred to as switching migration (random walk). Switching migration can occur dynamically and includes switching from radial to tangential migration or directed to non-directed manner, and vice versa. For example, tangentially migrating cerebellar GCPs in the external germinal zone place a leading and a trailing process oriented horizontally and then orient these processes vertically to the putative molecular layer from the cell body (Komuro and Yacubova, 2003). After a stationary period following the tangential migration, the GCs switch to radial migration and migrate to the direction of their descending processes (Komuro and Yacubova, 2003). Similarly, pontine nuclei neurons also switch their mode of migration from tangential to radial as they approach the region of the pontine nuclei. The leading processes of pontine nuclei neurons divert their direction radially and start radial migration. In some neurons of the pontine nuclei, the new-born process initially elongates radially and subsequently results in the radial migration of their soma (Hatten, 1990).

The basic mechanisms and principles of neuronal migration during development that are described above are general and similar for most of the neuronal types in the CNS. However, depending on the area of the developing CNS, diverse classes of neurons follow different strategies and may use distinct molecular cues and substrate during migration from their origin to their final position. Based on this, the following sections will focus on neuronal migration in the cerebellum.

THE NEURONAL MIGRATION IN THE DEVELOPING CEREBELLUM

Similar to the other regions of the brain, neuronal migration plays a substantial role in the development of cerebellar circuits (Hoshino et al., 2005). Relatively few cell types are aggregated to form the cerebellar gray matter, which includes the cerebellar cortex and the cerebellar nuclei (CN). The neurons that reside within the cerebellum are derived from two distinct germinal zones: the VZ and the rostral rhombic lip (RL). The VZ is the neuroepithelium of the alar plate of rhombomere 1 that will form the roof of the 4th ventricle. The neurons derived from the VZ includes PCs, Golgi cells, stellate cells and basket cells (Butts et al., 2014). All of these neurons are derived from neural progenitors that express pancreas specific transcription factor 1a (Ptf1a) and use GABA as a neurotransmitter (Hoshino et al., 2005). The cerebellar neurons derived from the RL at the dorsal edge of the cerebellar primordium, include the large neurons of the CN (which provide the output of the cerebellum), unipolar brush cells (UBCs), and the GCs (the most numerous cell in the brain; Elsen et al., 2013). All of these neurons originate from neuronal progenitors that express Atonal homolog 1 (Atoh1, formerly known as Math1) and use glutamate as their neurotransmitter (Manto et al., 2013).

The cerebellar cortex segregates into three layers: the molecular layer (stellate and basket cells), the PC layer (PCs and candelabrum cells) and the granular cell layer (GCs, Golgi cells, UBCs and Lugaro cells). The two most distinctive cells in the cerebellar cortex are the large PCs and the small GCs. PCs are the principal neurons of the cerebellar cortex, and the sole output of the cerebellar cortex projecting an axon to the CN. The molecular layer of the cerebellar cortex contains inhibitory interneurons, but is dominated by PC dendrites and parallel fibers which are the axons of GCs (Butts et al., 2014). PCs develop earlier and initially secrete sonic hedgehog (Shh) which is essential for proliferation of GCPs (Wallace, 1999). Under an intricate regulatory system, the appropriate numbers, migration and positioning of these cells is required in order for synapse formation and assembly of the cerebellar cortical circuitry.

The cerebellar cortex, which is the location of the most of cerebellar neurons, is compartmentalized and the cytoarichtecture is the most elaborately patterned circuit of all the CNS structures (White and Sillitoe, 2013; Beckinghausen and Sillitoe, 2019). The molecular expression patterns, afferent/efferent fibers, and birthdates divide the cerebellar cortex into an array of parasagittal stripes (e.g., Voogd, 1967; Hashimoto and Mikoshiba, 2003; Sugihara and Shinoda, 2004; Pijpers et al., 2006; Apps and Hawkes, 2009; Marzban and Hawkes, 2011; Bailey et al., 2013; Rahimi-Balaei et al., 2016) and

is further subdivided into four transverse zones (Sillitoe et al., 2005; Marzban et al., 2008; Marzban and Hawkes, 2011; Bailey et al., 2013, 2014; Rahimi-Balaei et al., 2016). The most extensive study of cerebellar cortex compartmentation was performed on PCs using zebrin II and phospholipase C beta 4, and resulted in a striking map of topographic stripes (Plcβ4) e.g., (Marzban et al., 2007; Kim et al., 2009; Bailey et al., 2014). The cerebellar cortical interneurons are also organized and restricted to the same zone and stripes pattern (Consalez and Hawkes, 2013). In addition, stripes of the cerebellar cortex align with the terminal fields of the two major cerebellar afferent types; mossy fibers and climbing fibers (Akintunde and Eisenman, 1994; Sugihara and Shinoda, 2004; Sugihara and Quy, 2007; Rahimi-Balaei et al., 2015; Sillitoe, 2016). Remarkably, an interesting birth dating study has revealed that the zone and stripe pattern is established before migration of cerebellar neurons. It was found that, the fate of PC topography is already specified according to their birth date during E10.5–12.5 (Hashimoto and Mikoshiba, 2003). Recently, it has been shown that the CN neurons (CNNs) are organized with molecular heterogeneity that may mirror the molecular complexity of the cerebellar cortex (Sugihara and Shinoda, 2007; Chung et al., 2009a; Sugihara, 2011).

Underlying the complex cerebellar cytoarchitecture with a few neuronal types is an intricate sequence of events in which neurons that originate from different germinal zones migrate via a complicated migratory pathway to their final position and establish elaborate cerebellar compartmentation and circuits (**Figure 1**). The cerebellar glial cells migration are not included in this review because of complexity and the molecular processes involved and need to be discussed in a specific focused review article.

Purkinje Cells Origin, Migration and Final Organization

PCs, which are key neurons in the cerebellar cortex networking, complete their final mitotic division at E10.5–12.5 in mice (Hashimoto and Mikoshiba, 2003, 2004; Minaki et al., 2008). Once in the postmitotic stage, they start a short distance radial migration along the radial glial fibers (scaffold/substrate) from their site of origin in the VZ (Rakic and Sidman, 1970; Yuasa et al., 1996; Hatten, 1999). PCs exhibit an elongated morphology consistent with axonogenesis at E12.5, which is directed toward the mantle zone where they accumulate as an irregular multilayer of cells called the cerebellar plate, or PC plate (PCP) at E14.5 (Miyata et al., 2010). However, PC axons project to the CN by around E16 (in mice; Sillitoe et al., 2009) and around E18 (in rat; Eisenman et al., 1991). During the early stage of PC migration, PCs initially have a leading apical neurite and trailing process, and the cell's position changes during cerebellar primordium expansion and morphological development (Hatten and Heintz, 1995; Sotelo and Dusart, 2009). At E13, the somata of cerebellar radial glial cells—the precursor of Bergmann glia—are aligned along the surface of the 4th ventricle, and extend processes up to the pial surface of the cerebellar plate. Along with translocation of the radial glial somata from VZ, the PCs undergo delamination and migration which is quickly followed by the detachment of the trailing process of the radial glia from the VZ (Yuasa et al., 1996). The migration of PCs along radial glia has not been documented in live preparations and this assumption is based on still images. However, it is believed that the newborn Purkinje cells from the VZ migrate radially, along the processes of radial glia. Recently it was shown that those Purkinje cells originate from caudolateral VZ migrate tangentially and cross the radial glial fibers but not along with them (Miyata et al., 2010; Sotelo, 2017; Schilling, 2018). With this evidence it can be speculated that despite the tangentially oriented PCs, some of these cells use radial glial fibers for their migration. The fast proliferating VZ neuroepithelium leads to prominent bulging toward the 4th ventricle and the caudal appearance of the cerebellar primordium seems oriented horizontally. However, radial glial fibers are connected from the neuroepithelium to the subpial surface and provide substrates for the PCs that originated from the caudal VZ.

After ∼E14.5 the PCP, a multi-cell-thick immature PC layer, is expanded in orchestra with the cerebellum development and aggregated in several PC clusters (PCCs) that become well established around E17.5 (Fujita et al., 2012). It appears that there is no migratory activity during this stage, but rather a small displacement of Purkinje cell groups following expansion of the developing cerebellum. The second wave of PC migration/displacement is initiated after formation of the PCCs. PCs disperse and arrange in a single regularly spaced monolayer during cerebellar expansion and simultaneously grow their axon and dendrites (Butts et al., 2014). During PC differentiation, they collapse their apical neurite (at P0) and form numerous short neurites at ∼P6 which develop ramified dendrites at ∼P8 (Armengol and Sotelo, 1991). In humans, PC progenitors start their migration from the VZ at stages18 and 19 of the development (∼44–48 days; Müller and O'Rahilly, 1990). They form a monolayer at 16–28 weeks of the gestation, and start the cerebellar enlargement with the development of more dendritic branches, which is associated with an increase in synapse formation (Müller and O'Rahilly, 1990).

It is not clear how PCs disperse from the cluster stage to the monolayer position (**Figure 1**). One of the most studied molecules that is involved in controlling PC migration is the Reelin pathway (Larouche and Hawkes, 2006). During mouse cerebellar development, the expression of Reelin (*Reln* mRNA and glycoprotein) is first detected at E13, along the dorsal cerebellar surface corresponding to the RL-derived cells and the nuclear transitory zone (NTZ; Fink et al., 2006). It has been shown that the delamination of postmitotic lateral Purkinje cells (at around E10.5) from the ventricular neuroepithelium and their initial migration is independent on Reelin signaling because at this time-point Reelin expression in the cerebellum has not yet started (Yuasa et al., 1993). By the first week of postnatal life, Reelin expression disappears from the deep areas but is maintained in GCPs, and the expression of Reelin may continue in some GCs of adult mice (Fink et al., 2006). Dispersal of Purkinje cells from the clusteric stage is

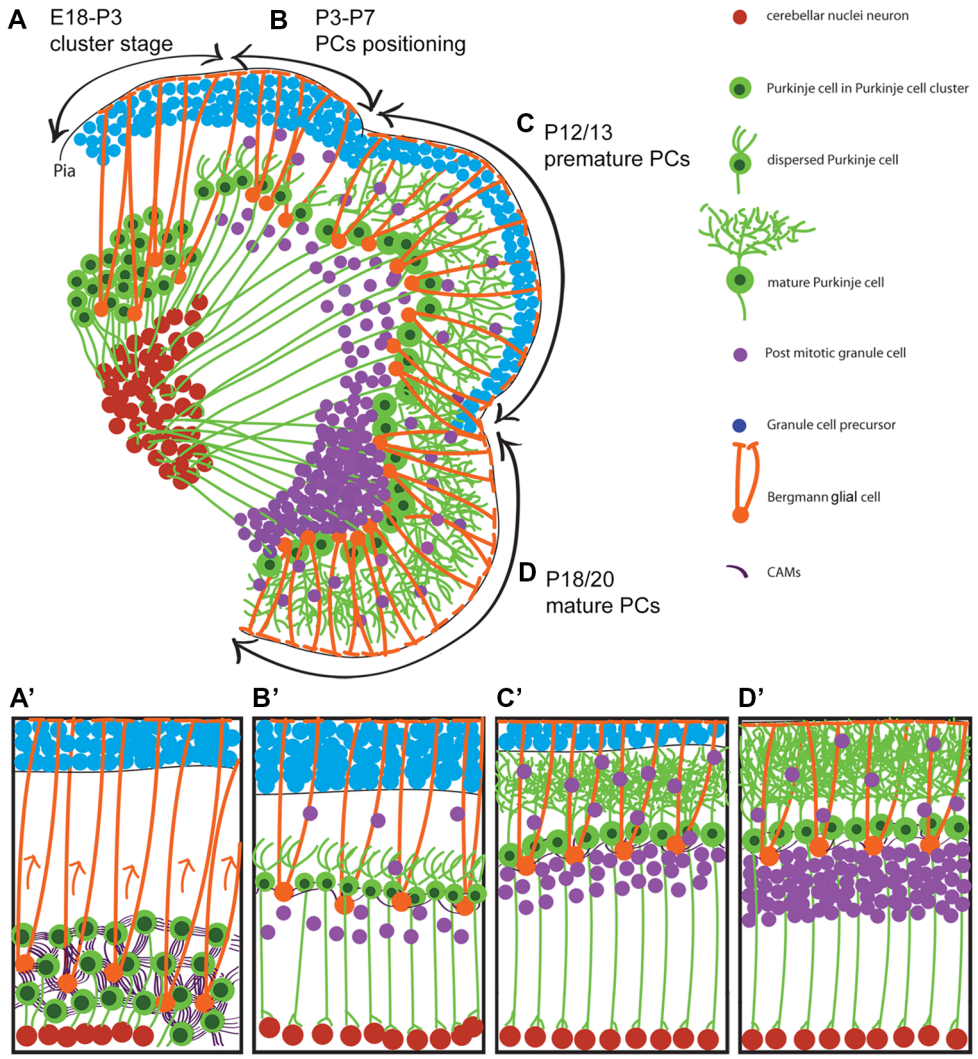

FIGURE 1 | Neuronal migration during postnatal cerebellar development. A schematic illustration of cerebellar development at sagittal section of postnatal mice. It shows that Purkinje cells (PCs) cluster disperse to establish PC monolayer and start maturation while granular layer form from external germinal zone (E18–P20). **(A,A')** Around E18 to P3, PCs are in clusteric stage and show high CAMs connections between PCs-PCs and PCs-Bergmann glial cell body which is located beside PCs during clusteric stage and extend their fibers to the cerebellar pia surface. **(B,B')** PCs in dispersal situation and positioning process (P3–P7) with less CAMs connections and show shortened Bergmann glial fibers. **(C,C')** It shows how premature PCs (P12/13) arborized (maturation process) while granule cell precursors (GCPs) migrate to the developing granular layer and become mature GCs. **(D,D')** At around P18/20 is end stage of the PCs maturation and GCPs inward migration. P, postnatal day; E, embryonic day; CAMs, cell adhesion molecules.

dependent on Reelin expression and its downstream molecules apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR). Reelin binds with similar affinity to ApoER2 and VLDLR. Disabled-1 (Dab1) which is a key molecule in the Reelin pathway, is expressed by Purkinje cells, as they settle underneath the Reelin-expressing cells of the external germinal zone (Fatemi, 2005; Miyata et al., 2010). Mutations in the *Reelin* gene (*reeler* mouse; D'Arcangelo et al., 1995), *dab1* gene (*scrambler* and *yotari* mouse; Howell et al., 1997; Sheldon et al., 1997), and targeted deletion of the genes for both *Apoer2* and *Vldlr* (Trommsdorff et al., 1999) all result in a similar phenotype of ectopic Purkinje cells due to a lack of dispersal from the cluster. These findings have placed Reelin, ApoER2, VLDLR and Dab1 into a common signaling pathway. It has been shown that the Reelin protein resembles extracellular matrix proteins that are involved in cell adhesion (D'Arcangelo et al., 1995) and regulate cadherin function via Dab1.

Cadherins are a group of transmembrane proteins that mediate cell–cell adhesion during tissue morphogenesis (Maitre and Heisenberg, 2013). Cadherin-6B (also known as cadherin-7) is overexpressed in Purkinje cell progenitors and is involved in guiding migrating neurons along neurites that express the same cadherin during their radial migration. These findings indicate that cadherin adhesive mechanisms are involved in neuronal guidance (Takei et al., 2000). It has also been

shown that Dab1 signaling controls the adhesive property of neurons to radial glia. The newborn neurons in the cortex of *scrambler* mice remain attached to the process of their parental radial glia during the entire course of radial migration and this abnormal neuronal-glial adhesion is highly linked to the positional abnormality of neurons in *scrambler* mice. Additionally, the phosphorylation of tyrosine residues of Dab1 regulates α3 integrin levels in migrating neurons and their timely detachment from the radial glial fibers (Sanada et al., 2004). Furthermore, direct interaction of phosphorylated Dab1 with other intracellular proteins such as members of Crk (adapter molecule crk also known as proto-oncogene c-Crk or p38) family might connect the Reelin pathway to integrin-mediated adhesion and migration of neurons (Ballif et al., 2004; Mayer et al., 2006). It has been shown that abnormal migration of neurons in *scrambler* mice is associated with an impaired detachment of neurons from clonally related radial glial cells. This abnormal neuronal-glial adhesion depends on α3 integrin signaling that is regulated by Dab1 (Sanada et al., 2004). However, a major challenge still remains: how do Purkinje cells from the clusteric stage disperse to their position in a monolayer?

It has been suggested that the PCC position changes during cerebellar surface development, which is extended rostrocaudally and mediolaterally and becomes arranged in a monolayer due to cerebellar expansion (Butts et al., 2014). In addition, GCs have been proposed to be a major player in the positioning of Purkinje cells postnatally (Jensen et al., 2002). However, several reports have shown that, Purkinje cells respond differently to different GC defects (such as hypoplasia, agenesis). In most cases, each affected cerebellum comprises several small percentages of Purkinje cells population, which are either in different ectopic locations, or arranged in the monolayer position. For example, a study of the *math1* null-mutant mouse in which the external germinal zone does not form, three populations of ectopic Purkinje cells have been described (Jensen et al., 2002). In the *scrambler* (mutation in *Dab1*, Reelin adaptor protein), the cerebellum is small because the size of the GC population is severely diminished by ∼80% and approximately 95% of Purkinje cells (not all) fail to complete their migration (Goldowitz et al., 1997; Reeber et al., 2013). Chemokine receptor 4 (*Cxcr4*) deficiency results in a lower number of GCs in the cerebellum and partially disorganized ectopic Purkinje cells (Huang et al., 2014), which is very similar to the phenotype described in *Weaver* mice (Smeyne and Goldowitz, 1989; Chen et al., 2009). It has been suggested that protein tyrosine phosphatase, non-receptor type 11 (Ptpn11) regulates formation of the laminar cerebellar cortex by controlling GC migration via the Cxcl12/Cxcr4 signaling (Hagihara et al., 2009), although removing *Ptpn11* in the external germinal zone has no distinct effect on cerebellar corticogenesis (Li et al., 2014). These results indicate that GCs are not the main player driving Purkinje cell organization in the cerebellum. Such an elaborate Purkinje cell monolayer organization cannot be explained by surface expansion and GC development. The development of this monolayer organization must be precisely regulated by active cellular and molecular processes rather than by a passive expansion.

Do PCs use any substrates or cells such as Bergmann glia cells to disperse from the clusteric stage to their final destination? Sudarov and Joyner (2007) introduced the role of GCs and Bergmann glia during formation of the base of each fissure (as an anchoring center) and proposed that this dictates the shape of the folia. Bergmann glia are defined as PC-associated astrocytes and are a specific type of astrocyte, which are zonally organized in the cerebellar cortex (Reeber et al., 2018). Bergmann glial cells originate from radial glia within the VZ. The radial glia transform to Bergmann glial cells during E14.5–E18.5 (in mice) under control of Ptpn11, which maintains the basal processes of the radial glia and relocates somata from the VZ to the nascent PCCs (Yuasa, 1996; Li et al., 2014). It is well documented that Bergmann fibers are associated with GCs in migration during cerebellar postnatal development, and this is the origin of the concept of glia-guided neuronal migration (Hatten, 1990). Recently, it was suggested that Bergmann glial cells are essential in cerebellar corticogenesis, especially through monolayer formation of Purkinje cells, dendritogenesis, migration of GCs, and circuit formation (Cajal, 1911; Rakic, 1971; Altman and Bayer, 1997; De Zeeuw and Hoogland, 2015; Leung and Li, 2018). Yamada et al. (2000) showed that not only are Bergmann glial cells associated with Purkinje cells in the adult cerebellum but they are also associated with Purkinje cells during their migration, dendritogenesis, synaptogenesis and maturation (Yamada and Watanabe, 2002). It is still controversial whether Bergmann glia regulate Purkinje cell monolayer formation through Notch-RBP-J signaling and notch ligand, Delta-like 1 (Komine et al., 2007; Hiraoka et al., 2013), since ablation of genes from Bergmann glia does not affect Purkinje cell monolayer formation while ablation of *Dner* (delta/notch-like EGF receptor containing) from Purkinje cells results in Bergman glia disruption (Eiraku et al., 2005; Tohgo et al., 2006; Greene et al., 2016). Bergman glia are rich in glutamate receptors and transporters [SLC1A3 (GLASTs) or the excitatory amino acid transporter, EAAT1] that are involved in Purkinje cell synapse formation (O'Hearn and Molliver, 1997). In fetal and neonatal stages, SLC1A3 is expressed ubiquitously in cerebellar radial glia or astrocytes and excessively in Bergmann glia at the postnatal stage (Yamada et al., 2000). It has been shown that the dendrites of the growing Purkinje cells ascend through the $GFAP^{+}/SLC1A3^{+}$ rod—like Bergmann fibers to reach the external granular layer (Yamada et al., 2000; Yamada and Watanabe, 2002). Studies on the *reeler* and *weaver* mutants has shown that SLC1A3 is down regulated in cerebellar astrocytes associated with Purkinje cells (Fukaya et al., 1999).

Reelin is also important for GFAP positive glial cell differentiation, process extension and orientation (Forster et al., 2006). In comparison to wild type mice, *reeler* mutant mice have unusual, numerous and heavily stained astrocytes with GFAP (Benjelloun-Touimi et al., 1985). Interestingly, in *scrambler* mutant mice, the cerebellum is small with no foliation, with GCs placed normally but their number reduced, and Purkinje

cell numbers decreased and placed ectopically (Goldowitz et al., 1997). Goldowitz et al. (1997) showed that the effects of Reelin on Purkinje cells could also be mediated indirectly by Bergmann glia. Although these studies indicate the role of the Reelin pathway in neuronal migration and Bergmann glia cell development, there is no evidence that Purkinje cells are using somal transduction or glial guided migration (Schilling, 2018). Therefore, it is possible that Purkinje cells utilize a different mode of migration in which they disperse passively with the assistance of a pulling force from the Bergmann glia by regulating cell adhesion molecules to form a Purkinje cell monolayer (**Figure 1**). This should be examined in future studies.

GABAergic Interneurons (Stellate/Basket and Golgi Cells)

Precursors of stellate and basket cells are generated within the VZ prenatally (when they express the paired homeobox gene, *Pax2*) and then migrate from the cerebellar plate to the developing white matter and postnatally (in mice) through the folial white matter while continuing to undergo cell division (Wefers et al., 2018). Thereafter, they migrate radially towards the molecular layer to accumulate at the inner border of the external granular layer and then migrate tangentially before settling at their final position within the molecular layer. A new study published by Wefers et al. (2018) document that the movement of cerebellar interneurons, basket cells and stellate cells, are highly directed and rerouted to the molecular layer during their transit through the nascent cerebellar cortex. They also showed that both the speed and directional persistence of basket cells and stellate cells are larger in the nascent GC layer than in the molecular layer (Wefers et al., 2018).

Golgi cell precursors are GC layer inhibitory interneurons and are born prenatally within the VZ. From the cerebellar plate these cells migrate to developing white matter while continuing proliferation during the migration until around P4 (Zhang and Goldman, 1996; Weisheit et al., 2006). During the perinatal development, Golgi cell precursors continue the migration through the developing folial white matter and terminate migration postnatally within the developing granular layer (Maricich and Herrup, 1999). In addition, a subset of Golgi cells are derived from the external germinal zone (Chung et al., 2011). These cells migrate within the white matter and become postmitotic postnatally and then migrate to position within the granular layer (Yamanaka et al., 2004; Wefers et al., 2018).

Although the mode and direction of migration of the GABAergic interneurons of the cerebellar cortex is a complex process and not entirely clear from origin to final position. However, based on the evidence the migration within the cerebellar cortex could be in the random walk mode.

Granule Cell Origin, Migration and Final Destination

The GCPs that originate in the RL (Atoh1 expressing progenitors) migrate tangentially through a subpial stream pathway, and over the cortical surface to form the external germinal zone, similar to the rostral migratory stream from the ganglionic eminences to the olfactory bulb (Komuro and Yacubova, 2003; Stenman et al., 2003; Machold and Fishell, 2005). Simultaneously, GCPs co-express *Pax6, Meis1, Zic1/2* and *Barhl1* while post mitotic (mature) GCs do not express *Atoh1* (Stoykova and Gruss, 1994; Ackerman et al., 1997; Miyata et al., 1999; Morales and Hatten, 2006). Although the existence of a substrate or scaffold in tangential migration has not been confirmed, it is possible that the pial meninges have this role as these processes are present underneath the pial surface (Komuro and Yacubova, 2003). In addition, the external germinal zone is unique among proliferative germinal zones of the CNS as it is adjacent to the pial surface rather than the ventricular surface. The cells in this layer are highly proliferative, generating an enormous number of granule cell progeny, thereby greatly increasing the thickness of the external germinal zone. In mice at E12.5 to E17, GCPs are born and migrate to establish the external germinal zone (postmitotic GCs typically sojourn for 1–2 days within the lower layers of the external germinal zone) and give rise to GCs during the first two postnatal weeks (**Figure 1**; Komuro et al., 2001; Wang and Zoghbi, 2001). In humans, the external germinal zone is distinguished as a distinct layer between 10 weeks gestation to 2 month postnatally and will disappear by about year one and a half (Marzban et al., 2015). GCs initially follow a tangential migration and after proliferation in the external germinal zone, the cells migrate radially. The GCs situated in the inner layers of the external germinal zone start redial migration along Bergmann glial fiber to form the granular layer while expressing *NeuroD1* (an early marker of the differentiated GCs). The expression of *Unc5h3* and *Pax6* continues throughout the life span (Komuro and Yacubova, 2003). The granule cells also change from a round cell to a more horizontal-oriented shape as they begin to extend axons tangential to the cortical surface. The CXCR4, a G-protein-coupled chemokine receptor, is broadly expressed in cells of the CNS and can mediate migration in response to its ligand, stromal derived factor 1 (SDF-1; also known as chemokine ligand 12, CXCL12). The CXCR4/CXCL12 signaling pathway is involved in the migration of GCPs in the rostral migratory stream from the RL. The alterations in this pathway result in the movement of GCPs toward deeper positions away from the meninges, i.e., the inward radial migration, to form the granular layer (Leto et al., 2016). In the GC migration pathway, Sema6A functions in the switch from tangential migration in the external germinal zone to radial migration along Bergmann glia (Leto et al., 2016).

These postmitotic GCs migrate radially inward from the external germinal zone and pass by the developing Purkinje cell layer, to generate the granular layer. The cells migrate along the processes of the Bergmann glia, which is only present in the cortex of the cerebellum (**Figure 1**). Electron microscopic studies have detected Bergmann fibers in the external germinal zone by E15.5 in mice, and by 9 weeks gestation in humans (Choi and Lapham, 1980). The radial migration of the cerebellar granule neurons depends on actomyosin of the leading-process which coordinates organelle positioning and adhesion receptor dynamics (Ballif et al., 2004). During cerebellar development,

DCX is strongly expressed by migratory GCs (as occurs in Purkinje cells) to mediate coupling of the nucleus to the centrosome (Gleeson and Walsh, 2000; Deutsch et al., 2010). Shh which is expressed by Purkinje cells plays a key role in GC proliferation, and may also provide a stop signal for GC proliferation and the beginning of the terminal differentiation as these cells migrate toward the source of Shh in Purkinje cell layer (Lewis et al., 2004). On the other hand, it is possible that the migration and maturation of GCs is not associated with Shh pathway signaling, but rather the subsequent loss of precursor cells from the external germinal zone limits the period over which postmitotic GCPs are generated (Lewis et al., 2004). The cellular and molecular mechanisms of cerebellar GC migration is reviewed by Yacubova and Komuro (2003).

Cerebellar Nuclei Neurons Origin, Migration and Final Destination

During the early stages of the cerebellar development *Atoh1*-expressing neural progenitors, which arise from the RL at around E9.5–12.5, give rise to the excitatory (glutamatergic) CN projection neurons (Manto et al., 2013; Marzban et al., 2015). The CNN precursors migrate tangentially from the RL through the rostral migratory stream to midway of the cerebellar primordium and then change direction toward the NTZ, a transient cell mass that is subsequently partitioned and organized to form the medial, interposed and lateral CN (Fink et al., 2006; Kurosaka and Kashina, 2008). During development of the glutamatergic CNNs, transcription factors *Pax6*, *Tbr2*, *Tbr1 and Lmx1a* are expressed sequentially within the neurons of the RL and the NTZ (Fink et al., 2006). It has been shown that *Tbr1* knockout mice have a similar number of CNNs, but the boundaries are not properly delineated (Fink et al., 2006). Neuroblasts that express Pax6/Reelin move radially to become Tbr2 positive cells (Fink et al., 2006). The formation of the dentate nucleus (the largest and most lateral cerebellar nucleus) begins by stage 20 (52 days in humans) when precursors (interneurons INs and projection neurons) migrate radially from the VZ and rostromedially from the RL (Marzban et al., 2015). In mice, projection neurons of CN originate from the RL and unipolar neuroblasts migrate in the subpial stream toward the NTZ under the guidance of both diffusible Netrin (a family of laminin-related secreted proteins) and Slit (an extracellular matrix protein; Fink et al., 2006; Guerrini and Parrini, 2010; Matsuki et al., 2013; Qin et al., 2017). Interestingly, a study using quail-chick chimeras has shown that the rostromedial end of the cerebellar primordium originates from the alar plate of the caudal mesencephalon (Hallonet and Alvarado-Mallart, 1996). By mapping the Ptf1a promoter with a reporter, it was shown that the VZ only gives rise to inhibitory neurons of the CN. Thus the CNNs are assembled in a coordinated fashion through integration of $PTF1a^{+}$ and $ATOH1^{+}$ lineages in local circuits that migrate from two different germinal zones (Leto et al., 2016). Nichols and Bruce (2006) hypothesized that the mesencephalic neural crest is the source of several migratory streams and it is the dorsal intermediate stream that gives rise to the neurons of the CN.

During brain development, in the majority of brain regions (including CN) the CXCR4/CXCL12 signaling pathway is the most important signaling pathway that regulates neuronal migration. This receptor first appears in immature neurons in the RL at E12.5 (Tissir et al., 2004) and the expression of this receptor continues in the RL-derived CN precursors during the rostral subpial migratory stream pathway to the NTZ. Their ligand CXCL12 is expressed simultaneously in the meninges overlying this migratory pathway. Similar to tangential migration of GCPs, this indicates the potential interaction of CXCR4- and CXCL12-expressing cells in the rostral migratory stream. This may facilitate the rostral migration of RL-derived neurons and also change the direction of the migration toward the NTZ. This occurs through the detachment of neurons in this region from the pial surface to descend toward deeper positions in the NTZ (Tissir et al., 2004).

Although there is some evidence for tangential migration of CNNs from the RL subpially to the midway of the cerebellar primordium, the mode of the migration and the substrate utilized during the change of the direction from the pial surface of the cerebellar primordium to the NTZ are not known. Furthermore, the mode of migration and the substrate pathway for the putative subset of mesencephalic derived cerebellar nuclear neurons are also not clear and need to be addressed.

Unipolar Brush Cell Origin, Migration, Final Position

UBCs project directly to GCs and amplify vestibular inputs to the cerebellum. In mice, the UBCs are generated from E13.5 to the early neonatal period (P0.5; Marzban et al., 2015). These cells originate from the RL and migrate through the developing white matter before settling in the GC layer of the cerebellar cortex (Hevner et al., 2006). The translocation of the UBCs from white matter to GC layer occurs between P3 to P10 and these cells sojourn in white matter for a period of time (Englund et al., 2006). Loss of the neurofibromatosis type 1 (*Nf1*) gene leads to imbalance in generating the appropriate numbers of glial cells, GC/UBC fate-specification/differentiation and GC/UBC migration (Kim et al., 2014). Studies have also shown a role for DCX in the neurogenesis and migration of UBCs (Manohar et al., 2012; Paolone et al., 2014). Furthermore, Wnt1 glycoprotein expression in the upper RL and UBCs is related to molecular identity and cell migration in cerebellar development (Hagan and Zervas, 2012). A previous study by Englund et al. (2006), reported that Tbr2 positive UBCs migrated from RL explants directly into the developing white matter of adjacent cerebellar slices (Hevner et al., 2006).

ANIMAL MODELS IN NEURONAL MIGRATION

The use of animal models is a powerful approach to understand both human disease and basic biology (Schofield et al., 2012). Several human developmental malformations have been attributed to defects in neuronal migration and have been

TABLE 2 | Mutant mice models with cerebellar neuronal migration defects.

Mouse model	Gene	Function	Defect	Reference
Reeler (like lissencephaly 2 or Norman-Robert syndrome in human)	*Reln*	Neuronal migration (Purkinje cells in cerebellum and pyramidal cells in cerebral cortex)	Ectopic Purkinje cell cluster (~80%), no foliation, cerebellar hypoplasia	Goffinet (1983), Yuasa et al. (1993) and Miyata et al. (2010)
Scrambler	*Dab1*	The Reelin-Dab1 signaling pathway involves in neuronal migration and also in lamination	Ectopic Purkinje cell cluster, no foliation, cerebellar hypoplasia	Chung et al. (2007, 2009b)
VLDLR/ApoE2	*VLDLR/ApoE2*	Reelin receptors involves in neuronal migration and also in lamination	Ectopic Purkinje cell cluster, no foliation, cerebellar hypoplasia	Reddy et al. (2011)
Src/Fyn	*Src/Fyn*	Downstream molecules of Reelin signaling pathway involves in neuronal migration and also in lamination	Ectopic Purkinje cell cluster, no foliation, cerebellar hypoplasia	Kuo et al. (2005)
Staggerer	*RORa*	RORa is a gene expressed only in Purkinje cells in the olivocerebellar circuit	Purkinje cells are small, ectopic, possess rudimentary dendritic arbors and their number is reduced by about 75%. All of the granule cells and 60% of the inferior olivary neurons die during the first postnatal month.	Hadj-Sahraoui et al. (1997)
Pten	*Pten*	Pten express in Bergmann glia (scaffold)	Ectopic Purkinje cells and Purkinje cell dendritic arborization	Yue et al. (2005)
SmoA2	*SmoA2*	Member of SHH pathway	Ectopic clusters of Purkinje cells with disorganized dendritic arbors and axonal collaterals	Armengol and Sotelo (1991) and Dey et al. (2012)
Naked Ataxic (*nax*)	*Acp2*	Lysosomal acid phosphatase 2	Excessive migration of Purkinje cells to the molecular layer, no Purkinje cell monolayer formation, cerebellar hypoplasia, reduced granule cells proliferation	Mannan et al. (2004); Bailey et al. (2013, 2014); Rahimi-Balaei et al. (2016, 2018) and unpublished data
p35/Cdk5	*p35/Cdk5*	Cyclin-dependent kinase 5 and its regulator, p35 involve in neuronal migration, proliferation and neurite outgrowth	Normal gross morphology, folia and lamination. Molecular layer with more cell density (GCs) and ectopic PCs in granular layer	Chae et al. (1997)
Weaver	*Girk2*	G protein-activated inward rectifier potassium channel 2	Beside PCs and GCs death, neuronal migration defect as a result of Bergman glia abnormality	Rakic and Sidman (1973)
Rp58	*Rp58*	GABAergic and Glutamatergic neuron development	Severe cerebellar hypoplasia and developmental failure of Purkinje cells, Bergmann glia and granule cells	Baubet et al. (2012)
CXCR4- and *SDF-1* deficient	*CXCR4*- and *SDF-1*(aka *CXCL12*)	The chemokine receptor 4 (CXCR4)–chemokine ligand 12 signaling pathway involve in neuronal migration and proliferation	Abnormal cerebellum, ectopic PCs, irregular external germinal zone	Ma et al. (1998), Larouche and Hawkes (2006) and Huang et al. (2014)
Astn or *Pex2*	*Astn* or *Pex2*	Genes for glial-guided neuronal migration	Ectopic granule cells precursors, abnormal Purkinje cell dendrite development, and external germinal zone present until late childhood cerebellum	Faust (2003)
BDNF	*BDNF*	Purkinje cells provide BDNF and promote granule cells precursors to differentiate and migrate along Bergmann glia fibers	Defects in cerebellar patterning such as ectopic granule cells precursors	Borghesani et al. (2002)
Rb/p107	*Rb/p107*	Survival of granule cells	Purkinje cells are disarranged with dystrophic dendrites	Marino et al. (2003) and Sotelo and Dusart (2009)
VPS18	*VPS18*	Disrupting multiple vesicle transport pathways to lysosomes	Neurodegeneration and impaired neuronal migration	Peng et al. (2012) and Davies (2013)
MDM2	*MDM2*	Link between p53 and Shh signaling pathways in granular neuronal precursors	Reduced levels of MDM2 and increased levels of p53 have small cerebella with shortened folia, reminiscent of deficient Shh signaling	Malek et al. (2011) and Gil-Sanz et al. (2013)
Tbr1	*Tbr1*	Cerebellar nuclei migration	Defect in medial cerebellar nuclei plus lateral and interpose	Fink et al. (2006)

confirmed in mouse models in which a gene mutation results in abnormal neuronal migration (**Table 2**).

In *reeler* knockout mice, the first manifestation of PCP malformation is at E14.5 and is prominent during cerebellar foliation at around E17.5 (Goffinet, 1983; Yuasa et al., 1993; Hadj-Sahraoui et al., 1997). This is mutation produces severe disorders in cellular migration throughout the brain and in the cerebellum it results in defects in Purkinje cell positioning, decreased proliferation and migration of GCs, and abnormality in foliation (Trommsdorff et al., 1999). The reduction and migratory defects observed in GCs could be due to Shh insufficiency caused by ectopically located Purkinje cells, which is far away from external germinal zone. The number of UBCs is also decreased and the cells are not positioned correctly (Trommsdorff et al., 1999). Interestingly, while there is no abnormality in the development of the NTZ in *reeler* knockout mice, the CN are significantly affected. The organization within the CN is especially disrupted in the lateral and medial CN. However, it has also been reported that alterations in the expression of the genes encoding the proteins in the Reelin signaling pathway do not change the morphology of the CN in mice. These components of Reelin pathway are cell surface receptor molecules VDLR/ApoER2, and intracellular signaling molecules Dab1, and tyrosine kinases Src and Fyn. In *Dab-1* mutant, *scrambler* mice, neurons show increased adhesion to radial glia which prevents them from reaching their final destination. These mice are ataxic and exhibit several neuroanatomical defects reminiscent of *reeler* mice. These findings indicate that abnormalities in the regulation of Reelin pathway result in cerebellar cortex anomalies which also result in defects in the development of CN (Fatemi, 2005).

In mice with a point mutation in the Lysosomal Acid phosphatase 2 (Acp2) gene, the result is a cerebellar defect with excessive migration of Purkinje cells to the molecular layer (Bailey et al., 2013, 2014; Rahimi-Balaei et al., 2016, 2018). We have recently investigated the role of Reelin-Dab1 signaling and its relationship to Erk1/2 (a member of mitogen activated kinases family) during Purkinje cell monolayer formation in the *Acp2* mutant cerebellum. Our findings indicate that down regulation of Reelin together with up regulation of phospho-Dab1 leads to the excessive and incorrect Purkinje cell migration in the *Acp2* mutant mice (under revision; Ashtari, 2017). In addition, it has been shown that the vacuole protein sorting 18 (VPS18), a core protein in intracellular vesicle transport, is involved in neuronal survival and CNS development. Genetic deletion of VPS18 leads to neurodegeneration and impaired neuronal migration as a result of disruption of multiple vesicle transport pathways that produce lysosomes. These findings indicate the importance of lysosomes in neuronal migration (Peng et al., 2012).

It is known that mouse double minute 2 homolog (MDM2), also known as E3 ubiquitin-protein ligase is a link between p53 and Shh. Using a p53 inhibitor it was shown that MDM2 is part of a signaling pathway in the development of GCs. It was reported that mice with reduced levels of MDM2 and increased levels of p53 have small cerebella with shortened folia, and Purkinje cells remained multi-layered and disorganized and exhibit stunted dendritic arborizations (Malek et al., 2011).

It has been shown that the lack of either *Astn* or *Pex2* (genes for glial-guided neuronal migration) produces a slowed migration pattern of GCPs which results in the formation of ectopic GCPs, abnormal Purkinje cell dendrite development, and the external germinal zone remains present until late childhood (Faust, 2003). Purkinje cells are an important source of brain-derived neurotrophic factor (BDNF) which promotes GCPs to differentiate and migrate along Bergmann glial fibers. Indeed, mice lacking BDNF have defects in cerebellar patterning such as ectopic GCPs (Borghesani et al., 2002). Finally, the deletion of CXCR4 leads to the premature migration of GCPs away from the proliferative zone of the external germinal zone, and small numbers of GCPs are found ectopically outside of the external germinal zone (Ma et al., 1998). Mouse models of fetal alcohol spectrum disorders and Minamata disease (a result of exposure to alcohol or methyl mercury during development) are also associated with deficits in GC migration related to interruption of a Ca^{2+}/cyclic nucleotide signaling pathway (Komuro et al., 2015).

Another study by Baubet et al. (2012) has shown that the ablation of 58 KDa repressor protein (Rp58) results in severe cerebellar hypoplasia and failure of Purkinje cells, Bergmann glia and granule cells to develop properly which leads to a delay in the formation of the primary fissure, number of folia and defective lamination of the cerebellar cortex. Marino et al. (2003) have investigated the role of Rb/p107 in the development of the cerebellum; and have shown that it is involved in the survival of granule cells. In Rb-deficient and Rb/p107 double mutants, Purkinje cells are disarranged with dystrophic dendrites. In Phosphatase and tensin homolog (*Pten*) mutant mice ectopic Purkinje cells are present (Yue et al., 2005). Similarly in mice with a genetic deletion of either smoothened (*SmoA2*, member of Shh pathway) or *Cxcr4*, ectopic clusters of Purkinje cells are present with disorganized dendritic arbors and axonal collaterals (Dey et al., 2012; Huang et al., 2014).

Early postnatal mice with a mutation in *Tbr1*, have abnormal morphogenesis of the medial CN suggesting that migration defects are associated with malformation of this region of cerebellum. It is important to note that *Tbr1* mutation is associated with the irregular formation of medial CN, as well as irregular formation of interposed and lateral nuclei. Interestingly, although there are some histologic malformations following *Tbr1* mutation, these changes are not correlated with the neuronal loss, cell death, or axonal abnormalities (Fink et al., 2006).

NEURONAL MIGRATION DISORDERS

Neuronal migration and positioning are critical processes during CNS development and circuitry formation, and defects in neuronal migration can lead to devastating brain diseases (Manto et al., 2013). It is well known that malfunctioning of the migratory process causes neuronal migration disorders (NMDs). NMDs are a heterogeneous group of birth defects with the same etiopathological mechanisms caused by the abnormal migration of neurons in the developing brain. This can result in neurological disorders with clinical manifestations including schizophrenia, autism, ataxia and epilepsy (Gleeson and Walsh,

2000; Nadarajah et al., 2003; Deutsch et al., 2010; Guerrini and Parrini, 2010; Demkow and Ploski, 2015; Marzban et al., 2015; Qin et al., 2017). The role of the Reelin pathway in neuronal migration has been extensively studied and in humans homozygous mutations in the *RELN* gene are associated with ataxia, cognitive abnormalities and cerebellar hypoplasia. In this context it has been also shown that the abnormal migration of cortical neurons is associated with reduced number of cortical gyri (lissencephaly). These results suggest an important role for Reelin in neuronal migration during the development. It should be noted that decreased levels of *RELN* expression have severe negative effects on the development of the human brain and may result in psychiatric diseases. For instance, patients who suffered from schizophrenia had reduced levels of *RELN* expression in the inhibitory neurons of their cortical areas. Additionally, decreased expression of Reelin has been observed in patients with other mental diseases, such as autistic-like disorders, bipolar disorder and major depressive disorder. Together these results suggest that Reelin has an important role in neuronal migration and synapse formation and deficits in Reelin expression may contribute to the pathophysiology of these disorders (Fatemi, 2005).

CONCLUSION

Neuronal migration is all about: where do neurons come from (origin), where do they go (neuronal migration pathway), and what are they going to become (differentiated neurons and positioning)? Given that different subsets of neurons may migrate long or short distances in different modes and directions before the positioning, there is no doubt that an accurate and precise regulation of neuronal migration is necessary in order to establish the appropriate neuronal architecture and perturbations during development can result in neuronal migration disorders. During the development of the brain, proliferative germinal zones have two important tasks which are: (1) to produce the right number of cells for the particular brain region (either too many or too few will result in abnormalities); and (2) to produce the right class of cells that need to migrate to the right position. The delineation of the regulation of these two tasks is a major goal of developmental neuroscience. In this review we have examined neuronal migration and its different modes with a focus on cerebellar cell types.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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