The role of cell adhesion molecules and Bergmann glia in Purkinje cell monolayer formation

By

Shahin Shabanipour Dehboneh

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Human Anatomy and Cell Science

University of Manitoba

Winnipeg, Manitoba

ABSTRACT

Purkinje cells (PCs) are large neurons in the cerebellar cortex with elaborate dendrites that receive inputs into the cerebellum. Being the only output of the cerebellar cortex, PCs project to cerebellar nuclei and control behaviors ranging from movement to social activities. Many cerebellar disorders are partly characterized by dysfunction/death of PCs without any effective treatments. Developing treatments relies on understanding early neurodevelopmental process. In this study, I investigate the migratory behavior of PCs in postnatal mice. Despite the current modes of neuronal migration that have been introduced so far, the main underlying mechanism of dispersing PCs from the cluster stage to the monolayer position is still unclear. **I hypothesize that Bergmann glia cells (BGCs) play a crucial role for PCs postnatal dispersal and monolayer formation**. To test the hypothesis, I used a mouse model of excessive PCs migration, naked ataxia (*nax*; *Acp2* $^{-/-}$), and investigated the expression pattern of genes which are potentially necessary to hold both cells together during migration. A group of most promising cell adhesion molecules were selected to study in *nax* and wt sibling mouse cerebellum. The expression of adhesion molecules was measured and visualized by western blot and IHC respectively. To clarify the contribution of protein values to the targeted cells, qRT-PCR was carried out on whole cerebellum and isolated PCs and BGCs from two different postnatal days. It is speculated that the characteristics of all four studied CAMs along with profile of their expression in BGCs strongly supports the possibility of BGCs-PCs connection at P2 and P7. However, the migration of BGCs as a carrier of PCs seems to be differently affected by the CAM members. Among all studied CAMs only the expression pattern of Ncam1 and Cdh4 is more in line with my hypothesis. Therefore, it is concluded that Ncam1 and Cdh4 are potentially involved in BGCs-PCs attachment, regulating the coupled migration and forming PCs monolayer in cerebellar cortex.

ACKNOWLEDGMENT

I cannot express enough thanks to my committee members for their continues support and encouragement in this journey: Dr. Kong, Dr. Del Bigio, Dr. Ghia and Dr. Dhingra. It was an honor to study under supervision of Dr. Hassan Marzban. His collaborative leadership and fatherly approach to address the issues during difficult times were really heartwarming. Completion of my project could not have been accomplished without the enormous support of my lab mates: Maryam and Azadeh, Technicians: Xiaodan and the manager of pharmacy FACS core: Christine.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACC: animal care committee

Ajs: adherent junctions

ASD: autism spectrum disorders

BGCs: Bergmann glia cells

BSA: bovine serum albumin

CALB1: Calbindin (calcium binding protein)

Cdh: cadherin

CCAC: Canadian council on animal care

CNS: central nervous system

DAB: diaminobenzidine

ECM: extracellular matrix

EGL: external germinal layer

FGFR: fibroblast growth factor receptor

FBS: fetal bovine serum

gcl: granule cell layer

HH: hedgehog family

HAV: histidine-alanine-valine

JAM: junctional adhesion molecule

MAPK: mitogen activated protein kinases

ME: mercaptoethanol

ml: molecular layer

nax: naked-ataxia

Ncam: neural cell adhesion molecule

NSC: neural stem cells

NMDA receptors: N-methyl-D-aspartate receptors

NRG: neuregulin

Pcl: Purkinje cell layer

PCR: polymerase chain reaction

PCs: Purkinje cells

PFA: paraformaldehyde

PSA: polysialic acid

SCA: spinocerebellar ataxia

SEZ: subependymal zone

SVZ: subventricular zone

TJs: tight junction

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 The Cerebellum

Cerebellum is a part of the brain involved in balance, coordination, and cognitive functions. Because of its well-defined neuronal organization, numerous researchers use the cerebellum to investigate neurodevelopmental processes in health and disease including neuronal migration (1). The cerebellar three-layer cortex consists of the molecular layer, Purkinje cell (PC) layer, and granule cell layer. The molecular layer, which is the most superficial layer located beneath the pia surface, contains dendrites of PCs, parallel fibres, GABAergic interneurons, and Bergmann glial fibres. The middle layer consists of a monolayer of PC soma which are intermingled with Bergmann glial cell bodies. And the third layer, as the inner most layer of cerebellar cortex adjacent to the white matter (comprised of PCs axons, the sole output of the cerebellar cortex), includes Golgi cells, unipolar brush cells and granule cells, (1). During normal cerebellar development especially corticogenesis in human, the migration of new emerged neurons such as PCs from ventricular zone and granule cells from external germinal zone is protracted from the embryonic period well into the 16–28 weeks of gestation and first year of life respectively. Cerebellum during this time is extremely sensitive to genetic, physical and chemical insults which implies the importance of studying early developmental process (2-4).

1.2 The function of PCs in cerebellum neural circuit and their characteristics in diseases

One of the highly complex neurons during early development of cerebellum are PCs. PCs are a group of large GABAergic neurons with highly dendritic arbors in cerebellar cortex that receive inputs into the cerebellum and have projection to modulate the excitation of cerebellar nuclei (5, 6). The output of the cerebellar cortex via PCs will control behaviors ranging from motor learning to social behaviors (5).

Many cerebellar-induced neurological disorders are partly characterized by dysfunction/death of PCs without any effective treatments. Studies show varying degrees of PCs degeneration in Autism spectrum disorders (ASD) (7, 8) and inherited and progressive ataxia in human (9-12). Surprisingly, the great plasticity of PCs helps these cells recover in one of the most common inherited ataxia, Friedreich's ataxia. The main cause of the Friedreich's ataxia in 95% of the patient with this condition is a homozygous GAA. TCC tri-nucleotide repeat expansion mutation within intron 1 of FXN gene which inhibits transcription of frataxin in mitochondria and leads to insignificant retrograde atrophy of PCs. Following the injuries caused by Friedreich's ataxia, PCs start intra-cortical branching and axon remodeling to re-establish connections among surviving cells. However, this process is not sufficient to fully recover the PCs' morphology. Several examples of pathological aberrations to PC morphology have been observed in other cerebellar disease as well, including axon torpedo formation and loss in cyto-architecture (13).Therefore, it is essential to find a treatment that rescues or replaces the damaged PCs. In the last decade, stem cell therapy, which is still passing clinical and preclinical tests, has become an interesting alternative to address the issue.

1.3 PCs development and improving treatments for neurodegenerative disorders

Stem cell/cell replacement therapy is an emerging field for treatment of neurodegenerative disorders such as ataxia. The main goal is to find the damaged or dying neurons and replace them with healthy ones. So far, the main source of postnatal neuro stem cells for transplantation and studies have been neurogenic regions such as subependymal zone or the dentate gyrus of the hippocampus. Cerebellum is another source of proliferative multipotent cells, but only available for a short period of time (14). In cell therapy, the injected cells into an injured cerebellum could hypothetically migrate to the desired locations, replace the damaged cells, establish connections

and restore the normal functions of the tissue/organ. This process is the foundation for the first series of cerebellar transplantation that was performed on the PCs progenitors derived from embryonic cerebellum (15). These studies were mostly focused on the protective effect of the stem cells on damaged PCs and how developing methods would help to generate new neurons from multipotent/polypotent cells. For example, in a mouse model of spinocerebellar ataxia (SCA) type 2, intravenous injection of human mesenchymal stem cells in the damaged region preserved the PCs and delayed ataxic symptoms (16, 17) Injecting neural stem cells (NSC) of the mouse increased motor activity, recovery of molecular layer thickness, and increased survival of PCs in this mouse model. Further studies revealed that the neuroprotection effect of stem cells begins upon contacting with the surviving neurons. Accordingly, NSC of a neonatal mouse was injected to adult mice cerebella with Machado–Joseph disease. The results following injection improved the motor coordination and increased level of neurotropic factors, decreased neuronal loss and reduced neuroinflammation in the damaged region (18). Generating new Purkinje cells have been performed *in vivo* and *in vitro*. The human iPSC (pluripotent stem cells) were successfully programmed into a Purkinje cells *in vitro* with 90% efficiency (19). Further studies even showed the possibility of producing mouse Purkinje cell progenitors In vitro which after injecting to cerebellum starts a maturation. However, only about 1% of the injected cells locate in pcl and the majority position in molecular layer which implies involvement of an underlying mechanism regulating PCs migration (15).

1.4 The current knowledge about Purkinje cell migration and their possible movement mechanisms

Therefore, improving the ongoing treatments requires a deep understanding of cellular and molecular changes during neurodevelopment. Migration plays a very important role in positioning

neurons from different proliferative zones, and correspondingly distinct lineages and genetic programs in a way to increase neural communication and potentially the efficiency of the nervous system. In addition, the positioning of different neurons during development varies with time which implies establishment of specific order in neurocircuit (19).

Neuron migrations are broadly classified as radial or tangential. Radial migrations include the glial-guided locomotion phase and soma translocation (glia-independent). There are a few unclassified migration modes as well which are featured with switching from tangential to radial migration or moving in a random directions(20). Varied combinations of these movements were suggested for different types of cerebellar cells. The only plausible explanation suggested for the migration of PCs so far was using radial glia fibres. At the beginning of the migration, the Purkinje progenitor cells reside in the ventricular zone. After a few postmitotic divisions, these cells attach to the radial glia fibres and organize in a plate at about E14 in mice (21). The PCs eventually move in a multilayer conformation (cluster) which manifest at about E14-E18 (22-24). The new wave of movement starts from P2-P3. At this time, the dispersal of the developing PCs occurs, which ends with residing PCs in a monolayer at P7(25). This model of migration, however, only explains the dependency of the PCs on radial fibres in general, while there is still little evidence whether the same mechanism is functioning the whole period of migration. Moreover, there are not many studies investigating whether this process is assisted by any other tools or cells along the way and the regulatory cascades presiding over these relationships are still unclear.

1.5 Development of Bergmann glia and their roles in promoting cerebellum neural migration

During development of the nervous system, the radial glial cells residing in ventricular zone of cerebellum are mostly responsible for radial migration of neurons. After birth, the majority of the glial cells in CNS transform to arborized astrocytes located in brain and spinal cord (26). The

Bergman glia cells in cerebellum are a type of astrocytes differentiated from radial glia progenitors (27). Their structural characteristics are similar to the nuclei of other astrocytes in the cerebellum of not only human but also in other vertebrates such as rat, mouse and monkey (28, 29). In rodents like rat, the soma of immature BGCs start migration at about postnatal day 0. During this time, the thin fibres of BGCs are recognizable. By P2 BGs are more mature and closer to the PCs. By P4, the developing BGs are located underneath the PCs and share similar spatiotemporal features (28). Later in the adult cerebellum, cell bodies of Bergmann glia mainly reside in the pcl and lower part of the ML(29). Similarly in mouse cerebellum, the BGs soma migrate along with PCs (25). The colocalization of BGs with PCs starting from P2-P3 is the first and probably the most important criteria of selecting a carrier cell in this study; a close contact between two cells whether physical or through chemical stimulation seems to be a prerequisite of a cell coupled migration. Besides, there are several evidence gathered over years of studying neural-glia interactions which support the idea that BGs soma, in a period of time, could serve as a migration mechanism for Purkinje cells (30). Both *in vivo* and *in vitro* studies showed that BGCs play crucial role in regulating movement of granule cell progenitors from external germinal zone (EGZ) to internal germinal layer (IGL). During postnatal days of development, the number of glia fibres entering EGZ increases (31) and BGs start interacting with granule cells by releasing glutamate to a large area between the fibres and immature granule cells (32). The glutamate interacts with of N-methyl-D-aspartate (NMDA) receptors located on migrating granule cells and regulates their movement (32). Additionally, the developing BGCs takes effect through the NRG–ErbB receptor signaling and FGF-FGFR1/FGFR2 signalling with granule cells in order to survive and maturation. Studies revealed that these neuro-glia interactions are mediated by not only the growth factors but also other effectors such as astrotactin and B1 integrin which are directly/indirectly related to the cell

adhesion functionality and ultimately affecting the neuron migration. BGCs have been suggested to share similar interactions with other neurons such as PCs and affect their development by promoting migration, glutamates homeostasis and maturation (15).

Further studies also suggested presence of intracellular junctions between BGs and PCs (33-35) that with variation would effectively set a specific spatiotemporal pattern for migration of both cells. Therefore, its very important to understand what type of junctions would mediate this interaction and investigate how their regulations would serve migration.

1.6 Types of cell connections/junctions in a multicellular organism

Cell–cell adhesion maintains the structural and functional integrity of multicellular organisms. Based on the structure and physiological properties of the adhesion the intercellular junctions are classified into tight junctions, adherent junctions, desmosomes and gap junctions (36).

1.6.1 Gap junctions

Gap junctions provide synchronized neural activity among glia cells and neural cells and between them. They also direct metabolite exchanges and presumably modulate cell growth, differentiation and organization of the cells (37). Besides, GJs are great mediator of the neural synaptic connections in the nervous system (38). Since the GJs are more featured with communicative roles than mediating strong physical connection between cells, they likely do not contribute to adherens and tight junctions.

1.6.2 Desmosomes

Desmosomes are a group of specialized and highly ordered domains on cell membrane that mediate cell-cell connection through their attachment to the intermediate filament cytoskeleton. In tissues and organs that are subjected to physical forces such as heart and skin, a combination of cadherin adhesion and intermediate filament are applied by desmosomes between cells to increase

the tissue resistance against mechanical stress. In the context of cell migration, desmosomes seem to take role in junctions where the cell-cell adhesion weakens. These observations have been reported in 2012 by Ewald et al group in normal, stratified, and elongating buds and by Shamir et al in 2014 in migrating cells that were expressing E-cadherin (39). However, despite the resemblance to classical cadherins and important roles in cell-cell adhesion, desmosomal cadherins do not express in the nervous tissue which excludes the family from my studies (40).

1.6.3 Tight junctions

Tight junctions (TJs) are mostly known for establishing a selective barrier against free diffusion between extracellular compartments across epithelial cell sheets (41). Their presence in the blood brain barrier for example limits the permeability of ions and molecules to the brain (42, 43). Depending on the number of cells involved in the sharing area there are two classes of tight junctions (2 cells in contact is called bicellular junctions and tricellular junction where three cells come together)(44). Tight junction structure is composed of tetraspan and single-span transmembrane domains and cytoplasmic domain which are linked to actin based cytoskeleton (45).The tetraspan TJs transmembrane proteins are claudin and occludin and the singlespan one is junctional adhesion molecule (JAM) (46). These domains besides controlling flux across both endothelial and epithelial cells, participate in regulating gene expression, cell proliferation and cell migration. As for the migration, studies showed that the occluding N-terminal domain of TJ's regulate neutrophil transmigration in the immune system. Suppression of TJs' transcription was also referred to as a common marker of promoting epithelial-mesenchymal transition (EMT) and increasing cell motility (47). Despite their close contact with other adhesion proteins and several reports about their contribution to migration, there are still not enough evidence to support their

direct enrolment in a strong physical connection between cells specially during neural development.

1.6.4 Adherens junctions

Adherens junctions (Ajs) are a superfamily of calcium dependent (cadherin based) anchoring junction molecules. They have three different morphological forms of puncta adherentia, zonula adherens, and fascia adherens. Their functional structure is composed of extracellular space which is in contact to a similar cadherin domain in other cells, and intracellular space attached to actin cytoskeleton (48). These compartments together transmit mechanical forces between cells. AJs are essential for the earlies stage of embryonic development (48); By regulating cell proliferating, polarity, shape, motility and survival, they play key roles in determining tissue dynamic and architecture (49). AJ molecules are broadly expressed in the nervous system; Especially during early development of CNS, their expression is critical for neural tube invagination and migration of neural crest cells from epithelial plate. AJs are not only mediating and regulating mechanical forces between cells, but are also capable of assembling TJs in the region (in epithelial cells) as well which emphasizes their multifunctionality and close relationship with other junctions (50). With over a decade of studies on these molecules, the current knowledge is still limited about their mechanism in different tissue and cells. Moreover, the answers to lots of the questions about AJ roles in development processes such as migration are not yet clear.

1.7 Expression of adherens junction CAMs in brain

The construction and functionality of the CNS networking are largely dependent on synaptic formation and connections between neurons, which are partly assisted and regulated by distinct cell adhesion molecules. In developing CNS, the migration of neurons is highly dependent on their interactions with their surrounding environments. The superfamily of CAMs is mediators of these

interactions with ECM and other cell surface molecules to fulfill their role in maintaining the tissue structure and regulating cell migration (51). They are also important for signal transduction, regulating cell survival and differentiation of progenitor cells (52). Their actions as an extrinsic signal and receptor of other molecules and transducing self-renewal and proliferation in progenitor cells of CNS is an important subject of cell programming and cell therapy (53). **CAMs are mostly categorized into four families: the cadherins, the immunoglobulin superfamily, the integrins, and the selectins** (40, 54).

1.7.1 Roles of cadherins in brain

Cadherins are a calcium dependant superfamily of transmembrane CAMs that mediate the cell-cell and cell-ECM connections through the interactions of the distinctive repeat sequences in their extracellular domains (40).They play several roles in the neural tube, neuroepithelial layer and boundary formation, synaptogenesis, axon guidance and regulation of neural stem cells. Cadherins have also been subjected to molecular coding that determines the specificity and identity of mediolateral and rostrocaudal Purkinje cell domains of PCs during development of cerebellar cortex in chicken, mouse and ferret (55). For example mutations in cadherin genes in mice will manifest cerebellar folia deficiency (55). Presence of calcium is an essential factor for proper function of cadherins and their concentration is strictly regulated in junctional area (56). Cadherins are able to dimerize in a cis (between two molecules on the same cell) or trans (between molecules on different cells) manner. The trans dimerization in a cell-cell attachment occurs between cadherins on opposing cells (57). The connections made between two cells can be mediated by cadherins of the same types (homophylic) or different types (heterophilic). The heterophilic binding are not broadly used in CNS and connection they establish are weaker than the homophilic ones (58) therefore the majority of the cells in CNS are expected to share the same cadherins for

connections. The Cadherins itself is categorized into 4 subfamilies including classic cadherins, protocadherins, desmosomal and unconventional cadherins (59). Desmosomal cadherins are the only member that does not express in neural tissue (40). Procadherins are cell adhesion molecules with varying number of extra cellular domain and divergent cytoplasmic domains that unlike other cadherins does not interact with catenins (58).

1.8 Classical cadherins

The classical cadherins in vertebrates are expressed in epithelial and neural tissues. They have five extracellular cadherin repeats, a transmembrane domain, and a highly conserved intracellular domain. The extra cellular domain interacts with opposing cadherin repeats offered by other cells in a homophilic manner, and the free Ca^{2+} in matrix determines their rigidity at the connection side (60). The cytoplasmic region of classic cadherins, on the other hand, are in contact with adaptor proteins to affect the cytoskeleton and cell-cell adhesion signalling (61).

Type I and Type II cadherins and their roles

Classical cadherins are classified into Type 1 and Type II. Both classic types share the common feature of interacting with actin cytoskeleton through catenin proteins inside the cells. However, they are different from each other in their adhesive interface. There are five members of cadherin type 1 (N-, E-, P-, M-, and R-cadherins) that are broadly distributed and segregated by embryonic germ layers or tissue type (62, 63). In CNS, the dynamic expression of Type I cadherins have been correlated to development of neural circuits (64), neural crest development (65) and axon guidance (66). Type II classic cadherins are structurally similar to type I , but they lack the histidine-alanine-valine (HAV) amino acid sequence in their extracellular domains of Type I (67) and share a very unique and different distribution in motor neuron pools of the spinal cord (40). The structural

differences between Type I and Type II classic cadherin members precludes their interactions between together (67) .

1.8.1 Classic cadherin type I

1.8.1.1 N-cadherin/ Cdh2

Cdh2 is a $Ca2^{+}$-dependent transmembrane glycoprotein and one of the main members of type I classic cadherins (62, 63). Cdh2 is broadly expressed in neuroepithelial cells during early embryonic and neonatal development, particularly in the nuclei and laminae, and neuroanatomical connections during late embryonic stages and early postnatal development (68). The earliest expression of Cdh2 in mice cerebellum has been reported at E14.5 and after birth was demonstrated at P7 and earlier (69, 70). Their orchestrated pattern of expression along with E-cadherins promotes neural tube invagination and their inhibition in neural crest cell leads to emigration of these cells during early development. Studies showed that Cdh2 deficiency leads to dislocation of cells in neural tube (65, 71, 72) . In postnatal cerebellum, the expression of Cdh2 is more localized to granule cells, PCs and cerebellar nuclei (67).

Cdh2 have essentially involved in establishment of left-right asymmetry (73) and synaptogenesis (74) as well as catenin-mediated processes related to learning and memory (75). Like other classical cadherin members, Cdh2 cytoplasmic domains are linked to cytoskeleton through symmetrical contact with catenins. Some members of cadherin binding proteins such as Ctnnb1also participate in development signalling pathways associated with Cdh2 (76). Therefore, disruption in β-catenin-Cdh2 relationship could negatively impact CNS development.

Role of Cdh2 in regulation of cell proliferation, survival and migration

As of the highly expressed cadherins in the nervous system, Cdh2 plays multiple regulatory roles to control cell proliferation of NS/progenitor cells. In one study, their overexpression in cells makes them exit the cell cycle faster than usual and enter differentiation phase through Hedgehog signaling. Their downregulation, on the other hand, increases the cell cycle through cytoplasmic β -catenin signalling and ultimately enhanced premature cell differentiation (77, 78). Cdh2 serves as a regulatory factor to warrant neural cell survival as well. Studies showed that in cooperation with pro-apoptotic protein Bim-related signaling pathway, Cdh2 enhances the survival of mouse spinal cord (79). Cdh2 is also involved in radial migration of cortical neurons. The proper radial migration of progenitor cells from germinal zones to pia surface of cerebrum and cerebellum determines the final morphology and function of brain cortex (80). In a Cdh2 Knocked out mice, the lack of Cdh2 expression disrupts this process and as a result the aberrant neural migration leads to disorganized cortex and lamination defects (81). These studies showed that also the knocking down Cdh2 increases premature neural migration in these cells. This suggest that neural migration is highly reliant on Cdh2 expression and variation from the required levels may change the fate of the migration. The regulatory role of Cdh2 in migration is fulfilled through membrane trafficking followed by activation of Rab GTPases-dependent pathways during corticogenesis (in mice). The Cdh2 mediated regulations can also change the orientation and polarization of the migration cells. In granule cells for example, aberrant expression of Cdh2 leads to their mispositioning and ultimately failing to maturate (82).

1.8.1.2 R-cadherin/ Cdh4

Cdh4 is one of the highly expressed classic cadherin type I in fetal brain and retina. Their structure and timing of expression is similar to typical classic cadherins like Cdh2 (70, 83, 84). The conserved internal C terminus domain of Cdh4 is linked to Ctnnb1 and cytoskeleton. The extracellular domain of Cdh4 was reported to be involved in cell-cell (homophilic) and cell-ECM (in glia cells of chicken) connection (85, 86).

Roles of Cdh4 in neurodevelopment

Cdh4 is involved in brain segmentation and neural outgrowth. Their interaction with PAX6 establishes adhesive codes which would guide pioneer axons (86). In cerebellum, the Cdh4 expression was demonstrated in PCs located on certain parasagittal domains and some granule cell raphe all after birth (87, 88). These stripes are reflecting heterogeneity of PCs and the key role played by Cdh4 as a migration regulator or cell junction mediator in subdivision organization (89). Cdh4 also present a high level of expression in early postnatal cerebellum than adults. In a study, total mRNA level of postnatal mice cerebellum was analyzed and the results though were not quantitative, clearly showed a strong mRNA expression in early postnatal (P1-P5) days which by P9 decreases and maintain the low level till P15 (87). The sudden drop of mRNA level might suggest that a series of regulations essentially take effect in the exact time frame of PC migration, from cluster stage to a PC mono layer (pcl). Although Cdh4 is not one of the mostly investigated classic cadherins in the nervous system, their lack of function may negatively affect social behaviours. Studies on Cdh4 knocked out (tm1b [EUCOMM] Wtsi allele mutation, homozygous) adult mice showed phenotypic/social manifestations such as decreased vertical activity and decreased startled reflex (90). Characteristics such as promoting neural growth, specific spatiotemporal expression in cerebellum and integrating abnormal motor functions and learning

behaviors in this model, which is similar to the symptoms of cerebellar conditions with damaged PCs, qualify Cdh4 as a potential regulator of migration for PCs (91-93).

1.8.2 Classic cadherin type II

1.8.2.1 Cadherin 8/ Cdh8

Cdh8 is one of the widely studied members of the classic cadherins in brain and cerebellum. They belong to type II classical cadherins that mediate calcium-dependent cell-cell adhesions (94). The In situ hybridization (ISH) based studies on mice CNS described the mouse cadherin-8 (mCad8) localization in both membrane and cytoplasmic regions (95). The molecular structure of Cdh8 is composed of three regional domains across cytoplasmic membrane, but lacks the extracellular domain sequence of HAV which is specific to classic cadherin Type I (96). There are 5 different motifs of extracellular Cdh8 which determines specificity of their cell-cell interactions, which are notably homophilic. However, their type II structure features selective heterophilic interactions as well which is a huge advantage in connections where neither cell is expressing the same adhesion molecule (97). In mouse cerebellum, the expression of Cdh8 (mRNA-based) is detected as early as E18 which continues in adult mice as well. Topological analysis of mouse neonatal and adult brain shows that the expression of Cdh8 is restricted to certain areas such as the limbic system, the basal ganglia–thalamocortical circuit, and the cerebellum which together establish neonatal neural circuits (68, 98). Studies on cerebellum showed that granule cells and certain domains of cerebellar cortex contain PCs that are Cdh8 enriched (95). The specific expression pattern of Cdh8 in PCs is very similar to the stripes of Cdh4 in parasagittal sections. This implies the involvement of Cdh8 in migration of PCs and their subdivision organization. However, apparently stripe pattern of Cdh8 is not limited to PCs' organization and it also participates in neuromere formation and segmentation of neural tube during embryonic stage. Accordingly, the Cdh8 positive and negative

regions are separated by borders corresponded with those of the neuromeres. The segmental expression pattern of PCs' from early days as P2 eventually turns into a more continues distribution of Cdh8 in adult PCs, but not in every region which gives rise to expression stripes. Cdh8 at later stages of development becomes more specifically localized in functionally connected regions such as brain nuclei and laminar structures. But the highlight of their expression pattern is at P6 in PCs, where they share interfacial space with BGCs (pictures though did not mark the Cdh8 expression in both cells) (95). Studies suggested that Cdh8 is involved in synaptic contacts and axon guidance during development (99). It is an essential adhesion molecule for forging neural circuits, maintaining their networks and neural fibres fasciculation (100). The correlation between certain neural disorders and abnormal status of Cdh8 implies the necessity having functional Cdh8 expression in CNS. Studies showed that the Cdh8 is related to learning disability disorders (64, 101-104), myoblastoma and autism spectrum disorder (105, 106). The knocked out Cdh8 mouse shows abnormalities in CNS functions such as aberrant synaptic transmission, abnormal retinal bipolar cell morphology (107). Most of the studies on Cdh8 so far were all based on ISH and our database still lack the cell based quantitative studies of Cdh8 either at mRNA or protein level in PCs and BGCs during early postnatal days. Therefore, it is necessary to furtherly study cerebellar cortex and to represent the Cdh8 expression changes in a more accurate way.

1.9 Immunoglobulin superfamily CAMs (IgCAMs)

The immunoglobulin superfamily CAMs (IgCAMs) represent one of the most ancient and diverse families of cell adhesion proteins. There are numbers of IgCAM molecules participate as a critical mediator of cell adhesions during neural development (40) Most them have heterophilic interactions which are not only with other IgCAM subtypes but also different classes of ECM proteins such as integrins. The members of IgCAMs are either attached to the cell membrane or

secreted to the ECM. The soluble form of IgCAMs are often the product of alternative spliced transmembrane protein genes and may modulate cellular attachments at the junction site (108). Two of the well known IgCAMs expressed in cerebellum particularly PCs are neuroplastin and NCAM subfamilies. Neuroplastin is mostly involved in synaptogenesis and synaptic plasticity. This adhesion molecule is the first member of IgCAM superfamily found to be associated with sub-divisional organization of PCs. Neuroplastin in PCs was first detected at P3; By P5, their expression becomes limited to small groups of PCs (stripes) and ultimately manifest distinctive puncta around these neurons at P7 (109). NCAM, on the other hand, is a typical transmembrane IgCAM molecule with a continues expression pattern in PC layer (110).

1.9.1 Roles of NCAM in cell adhesion and neural migration

Neural cell adhesion molecule (NCAM) is a glycoprotein located on the cell surface of various cell types, including neurons and glial cells (111). They are widely expressed in both embryonic and adult brains. Studies on normal mice showed the earliest expression of Ncam1 in metencephalon at E15 which extends to even adulthood (69, 112). The NCAM molecule is involved in neural outgrowth and cell differentiations through interacting with ECM integrins such as α3β1 and α6β1 integrins. They also promote EMT through affecting E-cadherin expression in brain tumor cells (113, 114). NCAM plays essential roles in migration of precursor cells and fasciculation and pathfinding of axons and synapses. The Ig like domain motifs of this molecule may act as an identifier of synaptic contacts for neurons (115) and ultimately increase synaptic plasticity (116). The unique feature of NCAM in IgCAMs superfamily is the 27 alternative mRNA splicing which resulted in producing three major protein isoforms: NCAM-120, NCAM-140, and NCAM-180. These isoforms are named based on their molecular weight. They share the same extracellular domain, but the transmembrane and cytoplasmic domains are different. These

domains are featuring the variety of functions for cells (117). For example, the intracellular domain of NCAM-180 is essential for the interaction of this isoform with dopamine D2 receptor and regulating dopaminergic signaling and behaviors (118). Moreover, neural outgrowth is mediated by homophilic NCAM-NCAM interaction (119). Their interaction on different cell surfaces is called Trans-homophilic binding and on the same cell surface is named cis-homophilic binding. Studies showed that stabilized NCAM-140 on lipid rafts (microdomains of plasma membranes that are selectively enriched in cholesterol, sphingolipids) (120) can enhance neurite outgrowth through both modes of the homophilic interactions (121, 122). NCAM is often conjugated to Polysialic acid (PSA) (long linear homopolymer glycan). PSA-NCAM is known as a neural precursor marker in NS/PCS of neurogenic regions and in certain regions of adult CNS such as SVZ, hippocampus, and rostral migratory stream (RMS) (123). The PSA in the nervous system is synthesized by two polysialyltransferases (ST8SiaII and ST8SiaIV) and mainly modulates functions of NCAM. Studies showed that PSA directs migration and differentiation of neural precursors during development (124). Since they are in close relationship with NCAM, the PSA dependent differentiation relies on cell-cell contact. PSA actions do not always serve a strong cell-cell contacts. Some studies showed that the negative charge of PSA seems to inhibit NCAM homophilic bindings and subsequently weaken the cell junctions during migration (125). PSA is also the highlight of NCAM enrolment in promoting PCs repair. They enhance regeneration of PC axons by overexpressing in astrocytes of the lesioned areas (126). Despite numbers of application in CNS, the PSA functions are still relied on existence of Ncam1 protein. Therefore, the lack of Ncam1 expression is expected to not only interfere neurodevelopment and differentiation but also halt any potential repair that could occur following microdamage's to the PCs. This is why knocking out Ncam1 in mouse (Ncam1 tm1cgn mutant HOM) adversely affects neural

differentiation and decreases the size of the brain (127). It also manifests abnormal social/conspecific interaction, abnormal anxiety-related response and increase exploration in new environment (90).

1.10 RATIONAL OF THE STUDY

PCs play an important role in regulating cerebellar neural circuits that control wide range of motor learning and social behaviours (5, 6). Treatment of neural degenerative disorders that are partly characterized by PCs' damage or loss (7-12) requires better understanding of their migration process. However, the postnatal migration of PCs has not yet been well studied. So far, the cortex physical expansion and attachment to the radial glia fibres in cerebellum were the only methods offered for PCs migration. Additionally, Reelin pathways and granule cells were proved to be involved in PCs migration as well. But these are only based on the beginning and the end of the migration and the important molecular pathways involved along the way and are not sufficient to justify the 7 postnatal days migration of PCs. What we do not know is the possibility of interconnecting with other cells along the way that could either assist or be the main migration mechanism for PCs. The appearance of BGS in postnatal 2 cerebellum (cluster stage) and its synchronized movement along with PCs to the pcl suggest a possible correlation between these two cells. Increasing amount of evidence verifies the possibilities of these correlations. BGs are astrocytes developed from radial glia progenitor cells. They assist granule cell migration and therefore the original concept of glial-guided neuronal migration is applied to them as well. A similar correlation is observed in ectopic PCs which are deeply affected by BGCs that are not differentiation. This suggests that the migration and organization of PCs are associated with BGCs' functions. Moreover, it is known that Reelin is an important asset of PCs migration. A majority of PCs in Reeler mouse (Reelin mutant) stop migrating, but still small percentage of them reach the

pcl. One possible answer is existing an alternative migration mechanism mediated by BGCs that managed to move proportion of PCs regardless of Reelin status. The ultimate methods of finding the PCs-BGCs correlation could be using a mouse model with irregular PCs/BGs migration. Therefore, a mutant mouse (*ACP2* mutation (*ACP2* $^{-/-}$(aka *nax*; naked ataxia)) featured with migration of PCs in molecular layer is used to serve the purpose. The so called excessive migrated PCs in *nax* cerebellum surprisingly and through an unknown mechanism are collocated with BGCs therefore making it an ideal condition to prove the PCs-BGCs coupled migration and understand their mechanisms. These evidences are suggesting criteria that qualifies BGCs as one of the key regulatory and mechanistically elements for migration of PCs. In this study I investigate what types of physical connections might be necessary to secure their coupled migration and understand how they are regulated during radial movement.

1.11 Hypothesis and objectives

1.11.1 Hypothesis:

I hypothesize that Bergmann glia cells (BGCs) play a crucial role for PCs postnatal dispersal and monolayer formation. In this regard, the aims of my study would be to prove the expression of essential Cell Adhesion Molecules (CAMs) in both cells and analyze how the change in their expression pattern may serve the idea of co-migration.

1.11.2 Objectives:

1- Demonstrating the location of early development of CAMs around PCs

2- Using qRT-PCR and Western blot to investigate the expression pattern of CAMs in tissue

3- Purifying PCs and BGs and analyzing CAM expression in the sorted cells

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal protocol

Animal ethics

All procedures were submitted and approved by 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) as well efforts were made to minimize the number of animals used and their suffering.

Animal model

The *Acp2* mutant (*nax*) mice in my study were obtained from transferring the *nax* embryos from Institute of Human Genetics in the University Medical Center, Georg-August University, Gottingen, Germany. The *nax* colony was established and developed in Genetic model centre of University of Manitoba. Mice were kept at room temperature with relative humidity (18–20°C, 50–60%) on a 12:12 light and dark cycle. The heterozygous mice were initially bred to achieve *nax* mutant (homozygote/heterozygote/wild type ratio was 25%:50%: 25%, respectively). Based on the phenotypic characteristics such as smaller size, alopecia (lack of hair), and ataxia in *nax* can be distinguished from the wild type littermates. The age of the studied mice ranges between P2 and P7. The *nax* phenotypes are hard to recognize at the early post natal days. Therefore, PCR based genotyping was performed to address the issue and avoid any bias.

2.2 Mouse genotyping

Genotyping of the mutant mouse was performed using AccuStartTM II Mouse Genotyping Kit (Quanta, Biosciences).

DNA extraction

Tail samples were added to a 70µl of Extraction Reagent. Then samples were heated to 95°C for 30 minutes followed by cooling in room temperature. Afterward an equal volume of Stabilization Buffer was added to the extracts. DNA was directly used to run PCR.

Polymerase Chain reaction (PCR)

The prepared DNA extracts were mixed with PCR reaction components to the final volume of 25 µL according to the kit instruction.

Table 1: Reagents for PCR

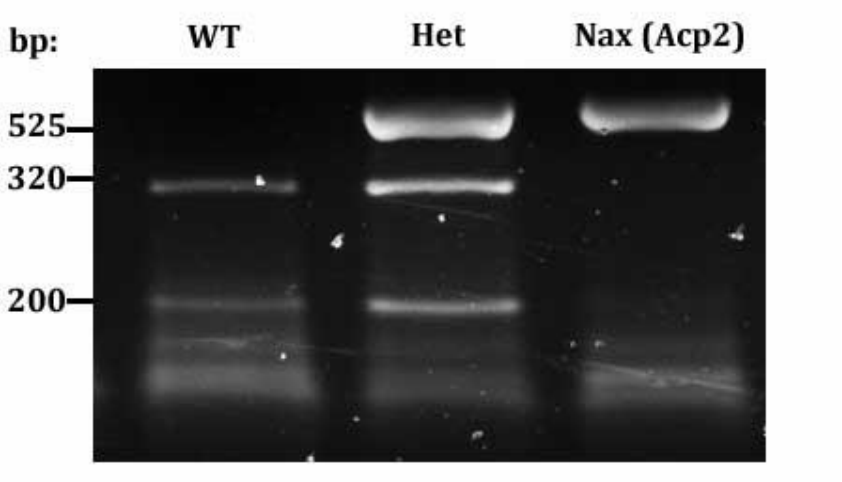
PCR Reaction Setup Component	**Volume**
AccuStart II GelTrack PCR SuperMix (2X)	12.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Nuclease-free water	9 µL
DNA Extract	2.5 µL
Final Volume (µL)	25 µL

PCR consists of series of repeated cycles with discreet temperatures steps as follow: (1) 5 cycles of denaturation step at 95˚C for 4 min (2) 32 cycles of annealing step at 56 ˚C for 45 sec (3) 1 min at 72˚C for extension following that the final extension at 72 ˚C for 10 min. Annealing temperature was selected based on the GC and AT content of each primer pair. Acp4F (5' GCACTCTGTGCCTTCTCCAT-3') and Acp4R (5'CTGGGAGATTTGGGCAACTA-3') primers were utilized in PCR reaction.

Restriction digestion with BamHI enzyme

The PCR amplicons from each sample were then mixed well with 2.5 µl of buffer and 1.66 µl BamHI restriction enzyme and incubated in a water bath at 37°C for 2hrs. Meanwhile, 2.5% agarose gel was prepared in 1x TAE buffer with 1µg/ml ethidium bromide. After incubation, the digested PCR products were run in the gel for an hour and the bands were visualised by a gel documentation system Fluorchem-8900 gel imager (Alpha Innotech Corp, San Leandro, CA, USA). Fragments of 525 bp were indicative of homozygous genotype, two fragments of 320 bp and 200 bp were indicative of wild type genotype.

Figure 1. Agarose gel showing different genotypes of *nax* mouse



Abbreviations: WT = Wild type, Het = Heterogeneous, Nax = Naked ataxia

2.3 Perfusion and sectioning

All mice were deeply anesthetized with 20% isoflurane, USP (Baxter Co. Mississauga, Ontario, Canada) diluted with propylene glycol (Sigma-Aldrich Canada Co., Ontario, Canada) in a desiccator. The mice were transcranial perfused with 15 ml of 0.1 M phosphate buffer saline (pH 7.4) and 30 ml of 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS). The brains were removed and post-fixed in 4% PFA at 4°C for at least 24 hours. For cryoprotection, the fixed cerebellum was washed in PBS by gently agitating on shaker for 30min-1hr. Then cerebellum was processed in 10% (2 h), 20% (2 h) and 30% (24 h) sucrose solution made with PBS. After sucrose processing, cerebella were transferred to the freezing blocks, oriented in Optimal Cutting Temperature (OCT) compound (VWR, Mississauga, Ontario, Canada) and frozen at -80°C for at least 1hr. Sagittal section the cerebellum were serially cut at a 25 µm thickness using a -20°C cryostat and collected on positive charged microscope slides precoated with gelatin. After finishing the sectioning, slide was kept in room temperature for 30 min and stored in -20.

2.4 Immunohistochemistry

To demonstrate the expression pattern of the CAMs in developing cerebellum, sections went through immunohistochemistry (IHC) and co-labeled with PCs, BGCs and the CAMs markers. During IHC process tissues sections on slide were washed with PBS 1X 3 times and incubated with NGS (normal goat serum 10% including 0.3% triton x 100) for an hour at RT. Then NGS was replaced by primary antibodies (Table 1) and incubated overnight at 4 °C. The primary antibodies were removed and sections were washed with PBS 1X 3times. Afterward tissues were fluoro tagged by goat anti-rat IgG (H+L) Cross-Adsorbed Alexa Fluor 488 (Catalogue No. A-11006, 1:1000 in blocking buffer, EMD Millipore) incubating with Alexa 488-conjugated goat-anti-mouse IgG and Alexa 549-conjugated goat-anti-mouse IgG secondary antibodies (Catalogue No.

A-11029 and A-11012 diluted 1:1000 in blocking solution, Life Technologies) at RT for an hour. The sections after washing were mounted with Fluor Save Reagent.

Table 2: Primary antibodies used in immunohistochemistry

Name of the Antibody	Dilution	Source
Rabbit polyclonal anti-calbindin D-28K antiserum CALB1	1:5000 in blocking solution	Catalogue No. 300 CEDARLANE
Mouse monoclonal anti-calbindin D-28K	1:1000 in blocking solution	Catalogue No. CB-38a CEDARLANE
Rabbit polyclonal anti-GFAP	1:1000 in blocking solution	Catalogue No. BT-575 Biomedical technologies Inc.
Mouse monoclonal anti-S-100 (β-Subunit) antibody	1:500 in blocking solution	Catalogue No. S2532 Sigma-Aldrich
Rat monoclonal anti-N cadherin (Cdh2)	1:100 in blocking solution	Catalogue No. MNCD2 Developmental Studies Hybridoma Bank Kyoto University
Mouse monoclonal anti-cadherin 8 (Cdh8)	1:100 in blocking solution	Catalogue No. CAD8-1 Developmental Studies Hybridoma Bank RIKEN institute
Mouse monoclonal_anti-NCAM	1:100 in blocking solution	Catalogue No.5B8 Developmental Studies Hybridoma Bank Columbia University

Table 3: Secondary antibodies used for immunohistochemistry

Name of the Antibody	Dilution	Source
Polyclonal Goat anti-rat IgG (H+L) Cross-Adsorbed Alexa Fluor 488	1:1000 in blocking solution	Catalogue No. A-11006 Millipore
Polyclonal Alexa 488-conjugated goat-anti-mouse IgG	1:1000 in blocking solution	Catalogue No. A-11029 Millipore
Polyclonal Alexa 549-conjugated goat-anti-mouse IgG	1:1000 in blocking solution	Catalogue No. A-11012 Millipore

Image Acquisition

The bright field microscopy of the IHC stained sections was performed by Zeiss Axio Imager M2 microscope (Zeiss, Toronto, ON, Canada). The Zeiss Light Sheet Z.1 microscope (Zeiss, Toronto, ON, Canada) equipped with a camera was used to visualize the immunofluorescent images from florescent labeled sections. The images were finally assembled into montages using an image editing software application Adobe Photoshop CS5 Version 12.

2.5 Western blotting

The protein analysis of CAMs using western blot was carried out on both *nax* and WT samples comprised of 3 cerebella for each strain. The collected samples covered with lysis buffer composed of protease inhibitor cocktail (Life Science, No. M250) and phosphatase inhibitor (Sigma Aldrich, No. P5726) almost double the volume of the tissue was homogenized by sonication. Then, protein concentrations were assessed using a commercial BSA kit (Bio-Rad No.5000121) and loading samples were prepared by adding the loading buffer (Tris-Hcl 60mM, glycerol 25%, SDS 2%, mercaptoethanol (ME) 14.4mM, bromophenol blue 0.1%, H_2O) to an appropriate volume of the sample to have the final concentration of $1\mu g/\mu l$. In order to perform electrophoresis, the loading

samples were heated in 100 degrees for 10 min and aftercooling down, 12 μl of each sample were loaded in the 8-12% polyacrylamide gel. 5 μl of precision plus protein were used as marker standard (Thermo Fischer Scientific, ON, Canada). Afterward, the proteins on the gel were transferred to PVDF membrane (Millipore, Mississauga, ON, USA) in transfer buffer (500nM glycine, 50mM tris-HCl, and 20% methanol) at RT O/N with the low voltage of 30V. For IHC, membranes were blocked for 1 h in 5% skim milk in TBS + 0.1% Triton X-100 (PBST). Membranes were incubated with primary antibody at 4 °C overnight with gentle agitation. Afterward, membranes were washed with TBST for 10 min (2-3 times change the washing buffer) and incubated with HRP conjugated goat anti-rat and anti-mouse secondary antibodies for 1 h at RT with HRP conjugated goat anti-rat IgG antibody (Millipore, AP136P) and HRP conjugated goat anti-mouse IgG (Catalogue No. AP308P, diluted 1:7500 in blocking solution, Millipore) (table 3). After the incubation and 10 min washing, the membranes were drained of washing buffer and positioned on a piece of Saran Wrap; Very quickly (avoid membrane drying) the following materials from ECL kit (Catalogue No. 170506; Clarity Western ECL Substrate) were mixed well in a 1.5 mL Eppendorf tube: 1ml Peroxide Reagent and 1ml Luminol/Enhancer Reagent. Membranes were soaked in the mixture for 60 sec. Right before exposing in the BIORAD ChemiDoc Imager the membranes were drained of the prepared ECL and wrapped in Saran Wrap. The protein bands from the blotting membrane images were quantified by Alphaease software. The house keeping protein β-actin was used as an internal loading control. The abundance of the targeted proteins from each sample were evaluated relative to the β-actin.

Table 4: Primary antibodies used for Western blot

Name of the Antibody	Dilution	Source
Rat monoclonal anti-N cadherin (Cdh2)	1:100 in blocking solution	Catalogue No. MNCD2 Developmental Studies Hybridoma Bank Kyoto University
Rat monoclonal anti-R-cadherin (Cdh4)	1:500 in blocking solution	Catalogue No. MRCD5 Developmental Studies Hybridoma Bank RIKEN institute
Mouse monoclonal anti-NCAM	1:100 in blocking solution	Catalogue No.5B8 Developmental Studies Hybridoma Bank Columbia University

Table 5: Secondary antibodies used for Western blot

Name of the Antibody	Dilution	Source
HRP conjugated polyclonal goat anti-rat IgG	1:7500 in blocking solution	Catalogue No. AP136P Millipore
HRP conjugated polyclonal goat anti-mouse IgG	1:7500 in blocking solution	Catalogue No. AP308P Millipore

2.6 Fluorescence activated cell sorting (FACS) of PCs and BGCs

Mice were selected from both wt and *nax* strains each at three different times (P2, P5 and P7). At the day of the experiment, mice were anesthetized, decapitated and the dissected brains were immediately transferred into ice-cold 1x Hank's balanced salt solution (HBSS, Gibco 14185-052) and washed by changing the buffer 3 times. Afterward, tissues were transferred to the dissection medium (1x HBSS containing gentamicin 10 μg/mL) for cerebellum isolation.

Cerebellum isolation

During this process the skull was held with fine forceps and cut through the calvarium from the lateral aspect of the skull in a line from the foramen magnum to the external acoustic meatus and inferior border of the orbital cavity. The skull base was removed and the thin layer of skull covering brain was peeled away. After exposing the brain, fine forceps were used to remove the cerebellum meninges from the lateral surface of the middle cerebellar peduncle and pons. The cerebellar peduncles were detached using scissors to isolate the cerebellum.

Cerebellum dissociation

The collected cerebella were placed and washed (centrifuge at 300 g, 4 °C and change supernatant) in cold Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12, Lonza 12-719F) 3x 1 min and incubated in trypsin (Gibco 15090-046) (37 °C) for about 12 min. The trypsin was inactivated with 10% FBS in DMEM/F12 and washed 3x 5 min.

Pellets were gently triturated with the 3.5 mL of DNase working solution (1 mL of DNase I stock solution [0.05% DNase (Roche 11284932001) + 12 mM $MgSO_4$ + 1x HBSS] in 500 µL of heat-inactivated FBS and 2 mL of DMEM/F12) to get a homogenous mixture of cells.

Immunohistochemistry of the dissociated cells and final sorting

The collected cells were counted (1×10^6) and blocked with NGS 10% in phosphate buffered saline (pH 7.4) with 0.1% sodium azide without triton X100 at RT for 15 min. The cells were then incubated in primary antibodies (Kirre Like Nephrin Family Adhesion Molecule 2 (Kirrel2) for PCs and Solute Carrier Family 1 Member 3 (SLC1A3) for Bergmann glia) for 30 min and washed 3x with staining buffer. Followed by incubation with secondary antibodies for another 30 min and counterstaining with DAPI, the final pellet of cells was resuspended in staining buffer. The staining buffer (1x PBS, FBS 1%, 25 mM HEPES, 1mM EDTA) was used for diluting antibodies and

washing steps during immunohistochemistry. Cell sorting was performed on BD FACS Aria-III cell sorter. Cells were sorted with 100-micron nozzle at the concentration of 15 million cells per ml in purity mode. PCs and BGCs were collected in 15ml Falcon tubes without any buffer.

Table 6: Primary antibodies used for cell sorting

Name of the Antibody	Dilution	Source
Mouse monoclonal anti-Kirrel2/NEPH3	1:200 in staining solution	Catalogue No. MAB2564 Clone # 318311 R&D system
Rabbit polyclonal anti-EAAT1/GLAST-1/SLC1A3	1:200 in staining solution	Catalogue No. NB100-1869SS Novus biologicals

Table 7: Secondary antibodies used for cell sorting

Name of the Antibody	Dilution	Source
Polyclonal Alexa488-conjugated goat-anti-rabbit IgG	1:500 diluted in staining buffer	Catalogue No. A-11008 Invitrogen
Polyclonal Alexa 647-conjugated goat-anti-mouse IgG	1:500 diluted in staining buffer	Catalogue No. A-11029 Life Technologies

2.7 mRNA extraction from purified PCs and BGCs and qRT-PCR analysis

The average number of sorted cells per each sample that were used for RNA extraction were about 50 K. Sorted cells were collected in tubes with the least volumes and immediately the lysis buffer from Qiagen RNeasy Plus Mini Kit was added to them. The rest of the steps was followed according to the instruction of the kit to have the high yield of RNA extraction and avoid

contaminations as much as possible (QIAGEN: Cat No./ID: 74134). The cDNA synthesis was performed by 0.25 ng of RNA in a qScript cDNA SuperMix kit (Catalogue number: 95048-100). The reaction mixture contained 10 µL PowerUp™ SYBR™ Green Master Mix (Catalogue No. A25742), 2 µL cDNA template and 1.2 µL of each forward and reverse primer in a total reaction volume of 20 µl. Thermocycling parameters were: 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 55°C for 60 s, 72°C for 60 s. All reactions were performed in duplicate, and gene expression values were normalized with respect to the reference gene, GAPDH and utilizing the $2^{-\Delta\Delta ct}$ method. Data are presented as means ± SEM.

Table 8. Primer sequences used for mRNA analysis

Gene	Forward sequence 5′ to 3′	Reverse sequence 5′ to 3′
Cdh2	CGGTTTCACTTGAGAGCACA	CTCGCTCCTGGAAGATGGTG
Cdh4	CAAGAAAGGGCAGACAGTGG	GTTGATGGGTGGGATGACC
Cdh8	TGCTGACGACCCAGTTTATG	ATTGCACGTGTGGCAAGTTC
Ncam1	TGGTTCCGAGATGGTCAGTT	GGATGGAGAAGACGGTGTGT
Gapdh	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG

2.8 Statistical methods

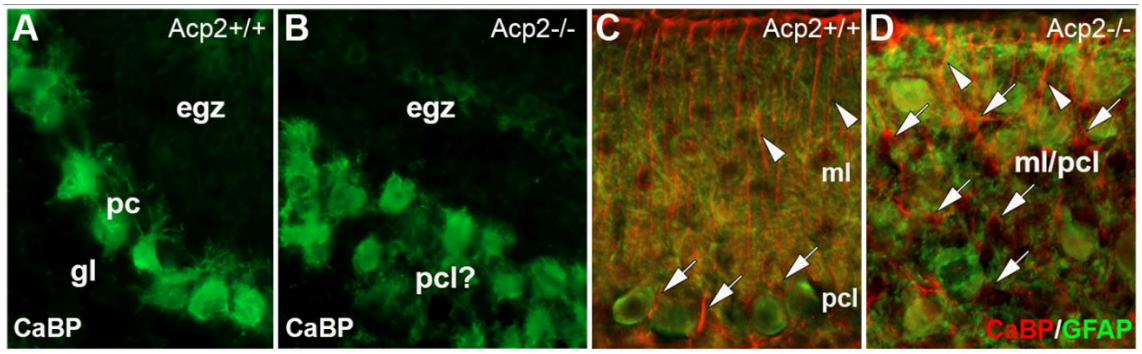
All experiments were repeated 3 times per each time point of the strain (n3). The raw quantitative (Western blot and qRT-PCR) results of each gene were then normalized to the hose keeping gene control (β-actin for Western blot and Gapdh for qRT-PCR data). The normalised results from each experiment were compared together based on two variances of time point and strain in ANOVA. The analysis and figure preparations were carried out using ANOVA multiple comparison testing in Prism Graphpad V7.05 and the $P \leq .05$ and $P < .001$ were considered significant.

CHAPTER 3: RESULTS

3.1 PCs excessive migration and BGC positioning in *nax* cerebellar cortex

In order to better demonstrate the effect of neuro-glia interactions in migration of PCs in our experiment a mutant mouse ($Acp2^{-/-}$; naked ataxia: *nax*) featured with excessive migration of PCs was used and the results were compared to wt littermates. The anatomical structure of the cerebellar cortex and the PCs organization in both *nax* and wt cerebellum were visualized by anti-CALB1 (CALB1; marker of PCs). The organization of PCs during early postnatal days (P6) which is close to their settlement in wt cerebellar cortex forms a linear shape which is called Purkinje cell layer (pcl) (Fig. 2A). In *nax* cerebellum, however, migration does not stop at pcl and by invading molecular layer and beyond the architecture of cortex becomes disrupted (Fig. 2B). The migrated PCs whether in *nax* or the wt cerebellar cortex share the same location with a group of specialized astrocytes, Bergmann glia cells (BGCs). In this study anti-GFAP was used to label and visualize the BGCs location in cerebellum. Glial fibrillary acidic protein (GFAP) antibody is one of the most typical markers of astrocytes which. To show the colocation of PCs with BGCs cell bodies and their extended fibres in both strains, transverse sections from cerebellum were double stained with anti-CALB1 and anti-GFAP (GFAP; marker of BGCs) as well. The differential organization of PCs/BGCs between *nax* and wt cerebellum are shown at later postnatal days as P15 (Fig. 2C, D).

Figure. 2. Localization of PCs and BGCs in *nax* and wt cerebellum

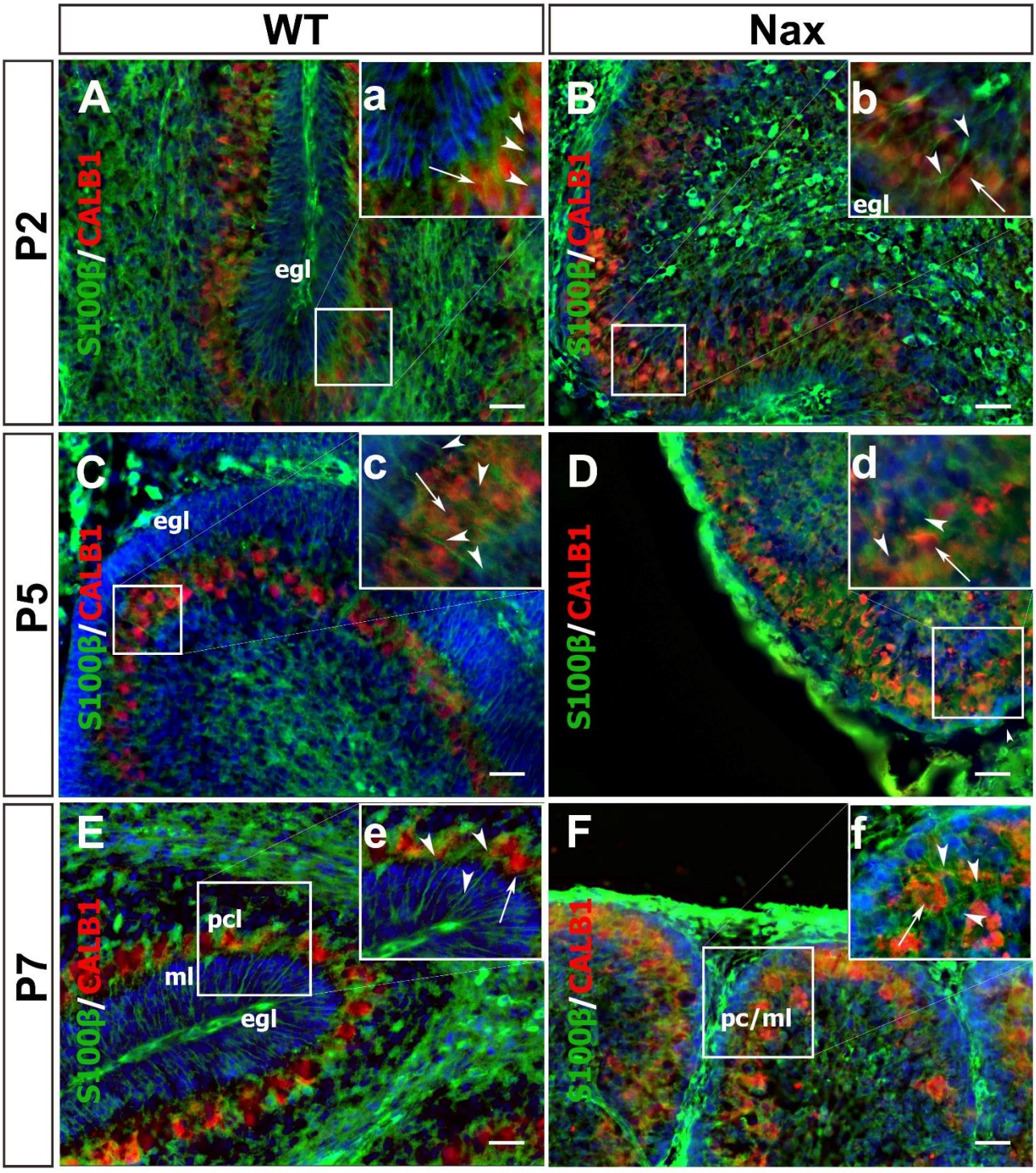


(A, B) Sagittal sections of Acp2$^{+/+}$ (wt) cerebellum at P6 immunofluorescence stained with anti-CaBP (green) shows monolayer formation of PCs in pcl **(A)**. Sagittal section of Acp2$^{-/-}$ (*nax*) cerebellum at P6 stained with the same antibody shows PCs are arranged to a multilayer conformation/cluster that extends to ML **(B)**. **(C, D)** Transverse section of Acp2$^{+/+}$ (wt) cerebellum at P15 double stained with GFAP (red) and CaBP (green) shows BGCs soma are aligned with PCs (arrow) in pcl **(C)**. The BGC bodies (arrow) are scattered around the cluster of PCs soma in molecular layer. The BGC fibres are in parallel in different length extending to the pia surface **(D)**. Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells; ML= Molecular layer; pcl=Purkinje cell layer.

Using GFAP marker, however, does not clearly indicate the location of the BGCs soma near the *nax* PCs. This is important especially about P6 or P7, when the excessive location of the Purkinje cells in the molecular layer is so disorganized that overlaps with the signals from fibres and soma of BGs. To confirm the BGC organization in cerebellar cortex, S100 IHC was used as an alternative BGCs marker. S100B is a calcium dependent protein mainly expressed in astrocytes, oligodendrocytes, and Schwann cells. During development of cerebellum, high levels of S100B is exclusively expressed in mature BGs and their precursors (128, 129). The soma and extensions of

BGCs labeled with anti- S100B antibody are demonstrated in *nax* and wt cerebellum along with PCs labeled with CALB1 at P2, P5 and P7 mice. P2 is the earliest postnatal day of PCs migration in which BGCs can be detected in cerebellum. In both *nax* and wt cerebellum the unbranched migrating PCs form a multilayer construction called cluster (Fig. 3A, B). The PCs at this stage are, however, less organized in *nax* cerebellum than the wt counterparts. The P5 cerebellum sections were used to provide an insight into the pre-settlement stages of PCs migration in both strains. The PCs at P5 are at the end of the cluster stage (Fig. 3C, D). PCs in wt cerebellum remained in pcl while in *nax* cerebellum they were positioned in further locations where ML was expected to be (Fig. 3E, F). In both strains, the BGCs soma are distributed among PCs in multilayer organizations at P2 and P5. The fibres of BGCs are extended toward the pia surface and at some points a second arm is developed in parallel with other fibres. The fibres are attached to the pia surface while their soma is collocated with PCs. At P7, BGCs are in close contact with the excessive migrated PCs in molecular layer while at the same day their wt counterparts are settled in pcl (where PCs are normally located) and stopped moving further (Fig. 3I-J). In both scenario the PCs are accompanied with BGCs.

Figure. 3. Colocalization of BGCs and PCs at P2, P5 and P7 *nax* and wt sagittal sections



(A-D) Sagittal section of cerebellum at P2, P5 and P7 were immunofluorescence labeled with anti-CALB1 (red) (CaBP; specific marker for PCs) and anti-S100β (green) (specific marker of BGCs). shows multilayer of PCs and the BGC soma. The BGCs fibre extension (arrowhead) are from PCs' (arrow) intercellular space toward pia surface at P2 **(A-B)** and at P5 **(C-D)**. High magnifications are shown in **(a-b)** and (**c-d**.**) (E-F)** The location of BGCs (arrowhead) is shown at P7 among PCs in pcl of wt cerebellum **(E)**. BGCs and their fibres are located in *nax* cerebellar sections in pcl/ml region close to egl **(F)**. The BGC fibres in *nax* cerebellum are not well organised as in wt cerebellum, but still are connected to the pia surface through their fibre extensions. Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells; pcl=Purkinje cell layer; ML= Molecular layer. Scale bars: A = 100 μm (applies to panels **A, B, C, D, E and F**); G = 50 μm (applied to panels **a, b, c, d, e and f**).

3.2 Analysis of mRNA-Protein level of CAMs in cerebellum and sorted PCs and BGCs

In order to understand how the interaction of the PCs with BGCs between them and their environments would impact their coupled migration, a set of most promising cell adhesion molecules such as Cdh2, Cdh8, Cdh4 and Ncam1 were selected and their expression was measured in the targeting cells.

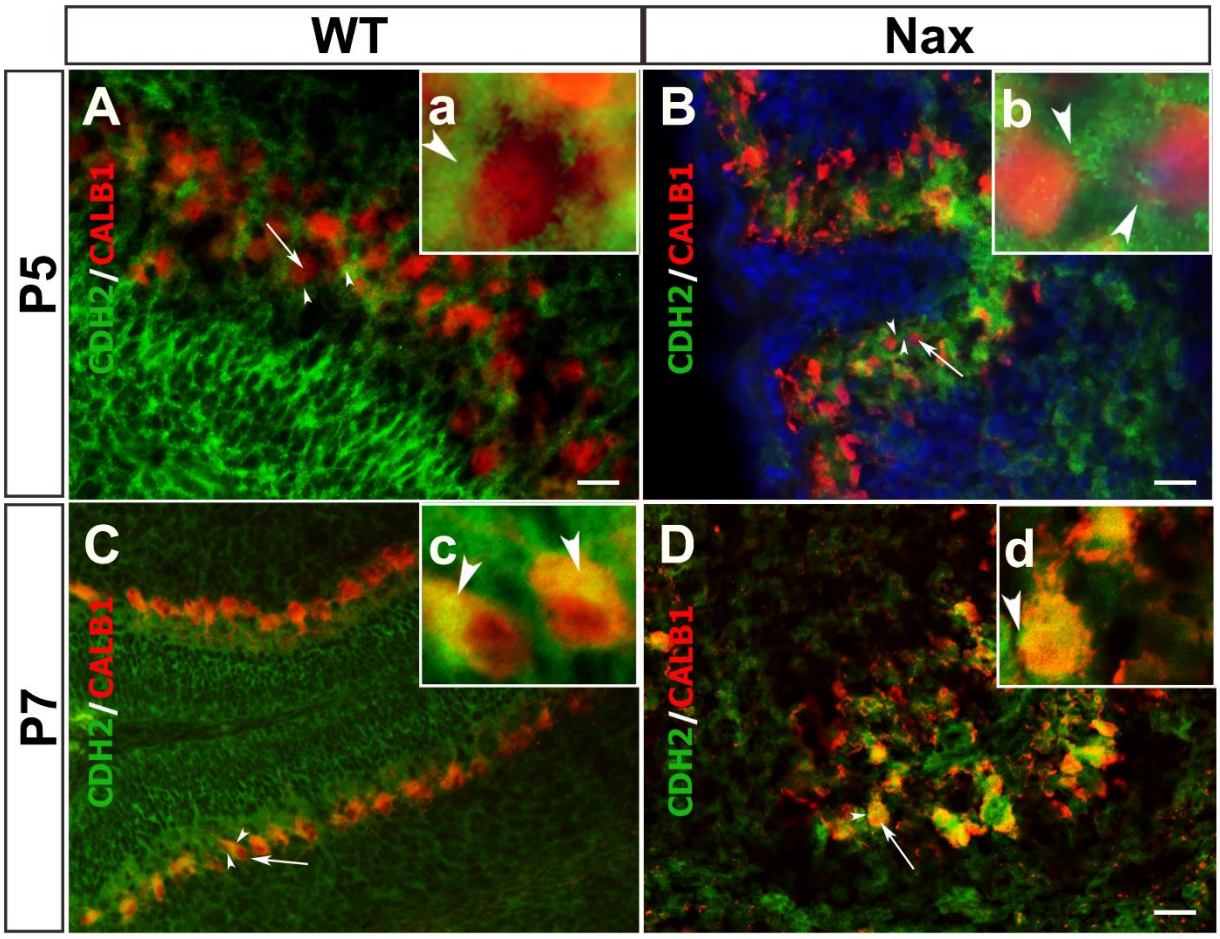
3.2.1 Cdh2 expression pattern analysis

Cdh2 is a member of classic cadherin type I and a typical adhesion molecule expressed during development of the nervous system (62, 63). In order to show the local expression of Cdh2 in neuro-glia clusters and pcl along with PCs, the *nax* and wt sections from P5 and P7 mice cerebellum were double stained with anti-CALB1 and anti- Cdh2 antibodies. The postnatal days 5 and 7 were selected as are close time points to the excessive migration of PCs. The expression

of Cdh2 in wt cerebellum is enriched in the cluster and mono layer of PCs at P5 (Fig.4A) and P7 (Fig. 4C) respectively. Cdh2 is expressed across the multilayer conformations of *nax* PCs as well. However, in *nax* cerebellum, Cdh2 signals are not as strong as in the wt tissue (*nax* P5; Fig. 4B, *nax* P7; Fig. 4D). To further investigate Cdh2 expression cerebellum, total Cdh2 protein level was measured in *nax* and compared with wt counterpart at P2 and P7. The Cdh2 protein expression in wt cerebellum showed an upward trend starting from P2 at a lower level compared to *nax* counterpart. The protein values in wt and *nax* ultimately became equal at P7. However, both *nax* and wt cerebellum alike did not show statistical significance between time points and strains (Fig. 5A). To understand how PCs and BGCs are contributed to the expression patterns of Cdh2, qRT-PCR was performed on the isolated PCs and BGCs, and the changing mRNA level of Cdh2 was analyzed between *nax* and wt cerebellum (Fig. 5C, D). Results showed that the Cdh2 mRNA in PCs is overexpressed in *nax* cerebellum. In both PCs and BGCs, the cellular expression is increased by time (between P2 and P7 wt BGC; $P<0.05$.) In *nax* cerebellum, changing Cdh2 expression by time was not considerable in BGCs. Moreover, the post mutation overexpression of Cdh2 gene in BGCs occurs only at beginning of coupled migration (P2) while at P7 the *nax*-wt differences are insignificant. Therefore, the general trend in BGCs proposes continues activity of a regulatory mechanism in *nax* BGCs, that lack of which in wt BGCs lead to increased Cdh2 at P7 and settling cells on pcl. In other words, both PCs and BGCs seem to acquire high Cdh2 transcription in *nax* cerebellum after mutation, but only one cell (PCs) is engaging up-regulatory mechanism for excessive migration. To gain a broader perspective of the Cdh2 transcription/translation in PCs and BGCs, qRT-PCR was carried out on whole cerebellum as well (Fig. 5B). Unlike the transcription pattern in PCs and BGCs, the total mRNA of *nax* cerebellum is lower at P7 than the corresponding wt. The negative trends of Cdh2 transcription between *nax*

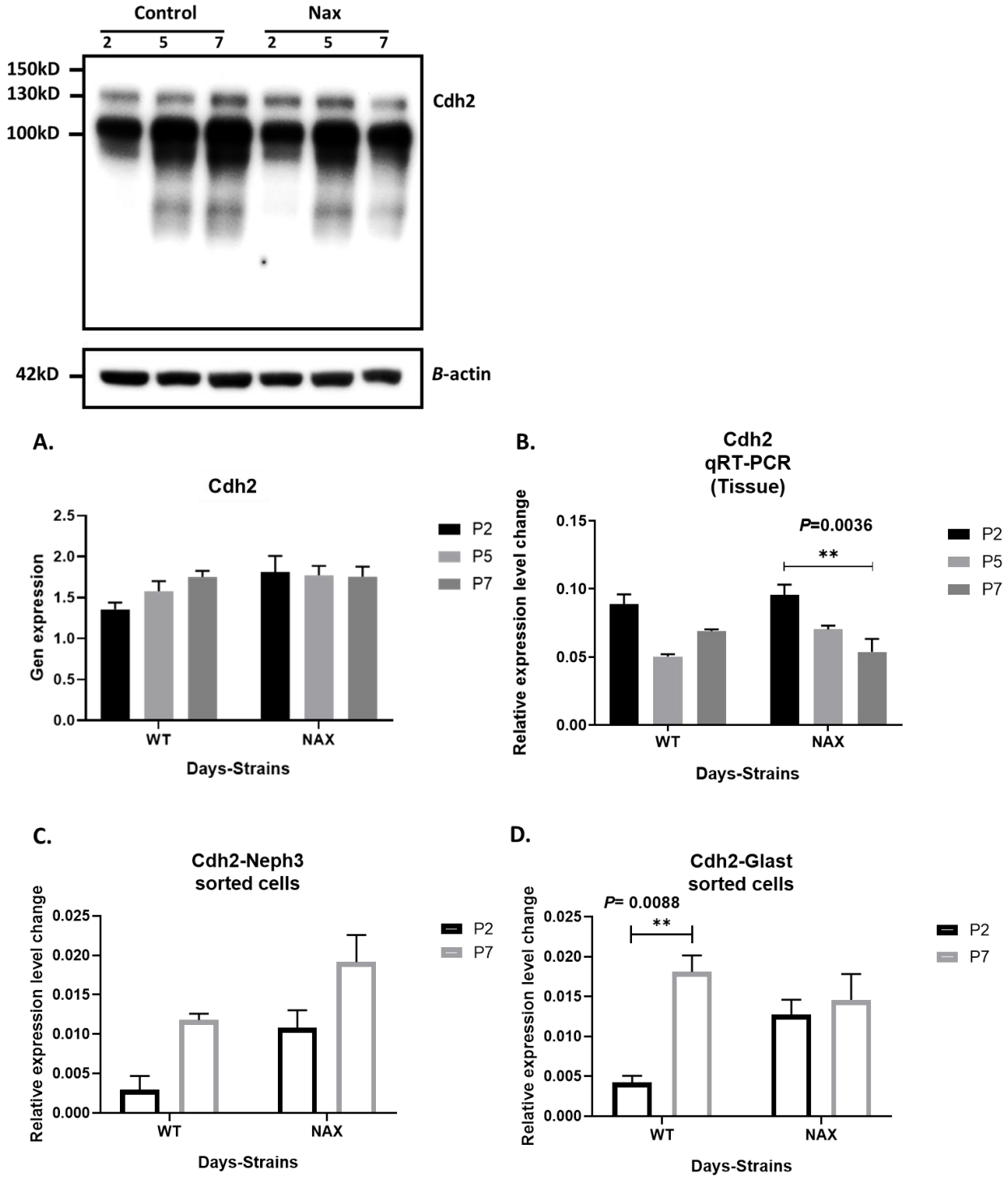
cells (PCs and BGCs) and *nax* tissue at P7 may suggest that a large amount of the this Cdh2 across the tissue could be produced from a specific type of cells such as PCs and BGCs. Not mention that according to the IHC images part of the broad scale of Cdh2 expression could be from granule cells which migrate inward from EGL to IGL during the time. The pattern in *nax* total mRNA, however, is leveraged in protein expression without any variations among ages and between strains meaning the possibility of translation enhancers between P2 and P7 in *nax* cerebellum.

Figure. 4. Colocation of PCs and Cdh2 in wt and *nax* cerebellar cortex



(A-B) Double staining of *nax* and wild type sibling mouse sagittal sections with anti- Cdh2 (green) and anti-CALB1 (red) shows the signals of Cdh2 (arrowhead) surrounding the PCs (arrow) at P5. The immunofluorescent signals from PCs and Cdh2 does not overlap at P5. Cdh2 is expressed in granular layer of wild type cerebellum at P5, but in *nax* the granule layer signals are not clear. **(C-B)** Double staining with anti- Cdh2 (green) and anti-CALB1 (red) in sagittal sections of *nax* and wild type sibling mouse cerebellum at P7 shows the intercellular (between PCs) and overlapping signals of Cdh2 with the soma of PCs. The Cdh2 signals is extended from pcl to the granule layer in wild type cerebellum, but in *nax* cerebellum it is not clear weather the signals are emitted from granule cell layer or the PCs in pcl. **(a-d)** Higher magnification of the local Cdh2 expression. Abbreviations: PCs=Purkinje cells. Scale bars: A = 50 μm (applies to panels **A, B, C and D**).

Figure. 5. The upregulation of Cdh2 mRNA in wt and *nax* cerebellum at P7 only occurs in PCs



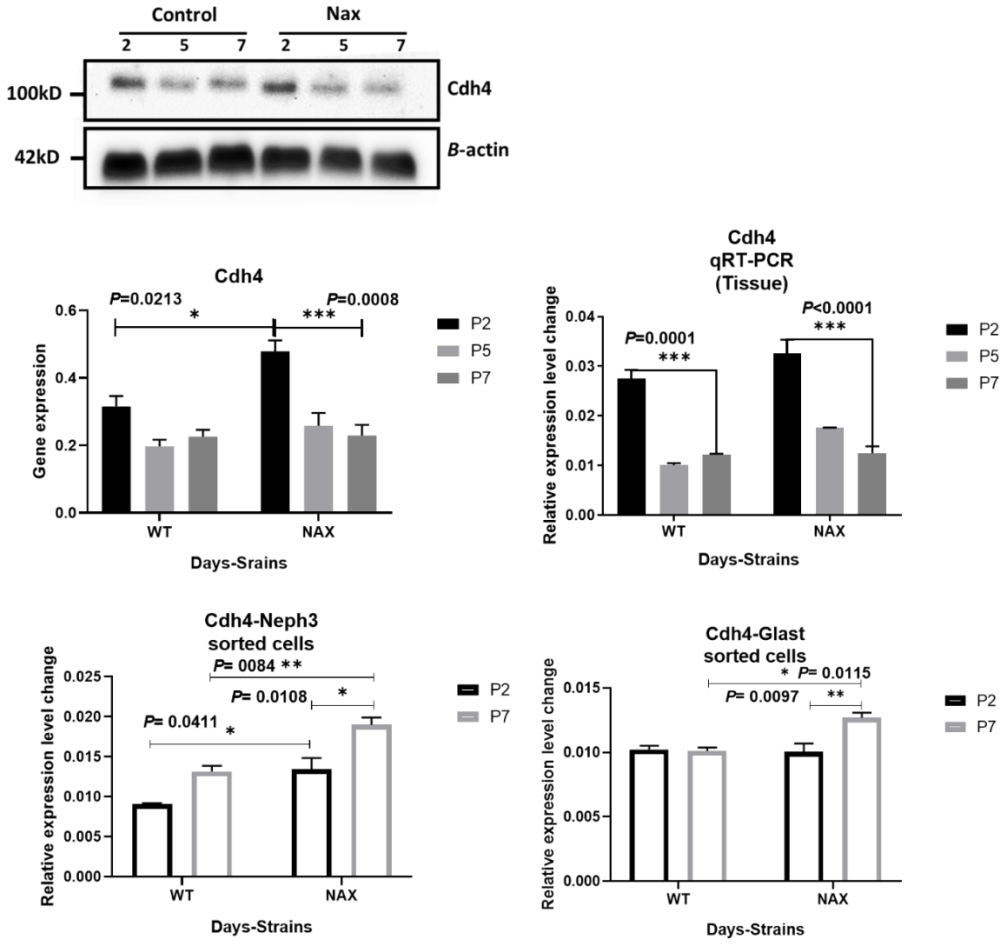
A) Cdh2 expression in cerebellum is measured by western blot at P2, P5 and P7; The variation between time point and strains are not statistically significant. **B)** qRT-PCR of mRNA collected from isolated PCs and BGCs from *nax* and wt cerebellum at P2 and P7; Cdh2 transcription at P7 is higher in *nax* PCs than wt PCs. In BGCs mRNA share similar values in *nax* P2 and *nax* P7; The *nax* BGCs values are incomparable with corresponding wt mRNA which shows significantly higher value at P7 than P2 (P <.001). (**C, D)** qRT-PCR of total tissue is downregulated in *nax* cerebellum between P2 and P7. This experiment was repeated using three different litters for each postnatal day in wt and *nax* siblings (wt; n=3 and *nax*; n=3). The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using multiple comparison ANOVA (P-value ≤.05 was considered as statistically significant.) Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells.

3.2.2 Cdh4 expression pattern analysis

The distinctive roles of Cdh4 as members of classic cadherin type I has been frequently mentioned in segmentation and neural outgrowth of brain (86). To measure the differential protein expression of Cdh4 at P2, P5 and P7 in both *nax* and wt cerebellum, western blotting was performed using anti- Cdh4 antibody. Our finding showed that the expression of Cdh4 in both strains is downregulated between P2 and P7. P2 is the only time point during cerebellar development that Cdh4 expression is higher in *nax* than wt ($P<0.05$) (Fig. 6A). In order to investigate if the same pattern is applied in PCs and BGCs, the mRNA from targeted cells was extracted and analyzed with qRT-PCR. Results showed an upward trend of the Cdh4 transcription in wt PCs between P2 and P7 (Fig. 6C, D). In *nax* PCs/BGCs, Cdh4 mRNA transcription is not only increased by time but also acquire higher values than the wt counterparts. The only exception is mRNA values of

BGCs at P2 which neither increased by time nor changed after mutation. This pattern indicates a Cdh4 up-regulatory mechanism in BGCs that is only triggered by mutation at P7, not P2. To further clarify the connections between our data, qRT-PCR analysis was carried out and total Cdh4 transcription was evaluated in cerebellum from both strains (Fig. 6B). The total expression of Cdh4 across both strains of cerebellum is decreased from P2 to P7, while the trend for cellular mRNA values are negative. The inverse pattern of values between *nax* PCs/BGCs group and *nax* mRNA-protein group indicates that the majority of Cdh4 expression in cerebellum is dedicated to the targeted cells at P7. Therefore, it is indicating an indirect confirmation of mRNA analysis data collected on each cell type.

Figure. 6. Cdh4 is overexpressed at P7 in PCs and BGCs of mutant mouse cerebellum



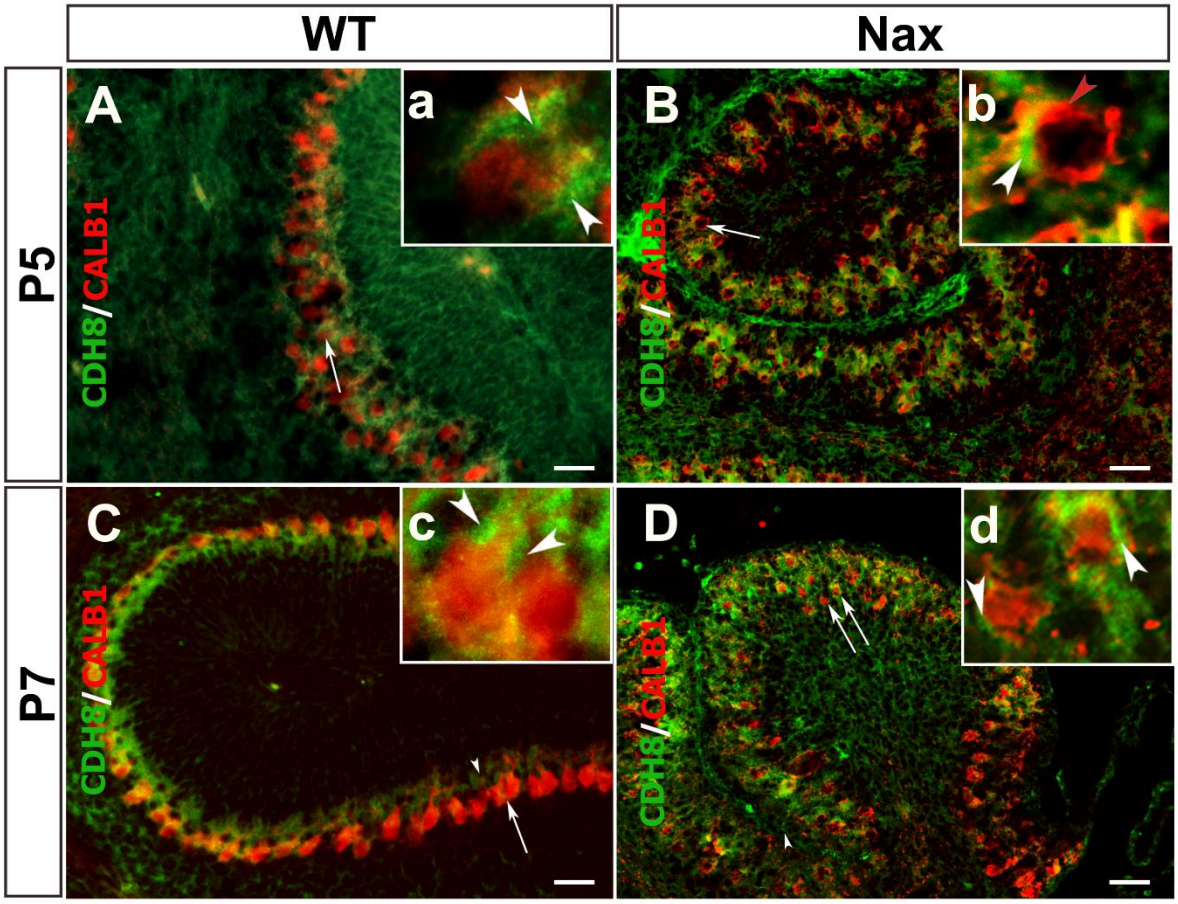
A) Cdh4 expression in cerebellum is measured by western P2, P5 and P7; protein expression in wt cerebellum is downregulated between P2 and P7; In *nax* cerebellum Cdh4 expression is significantly decreased between P2 and P7 (P <.001). **C, D)** qRT-PCR of mRNA collected from isolated PCs and BGCs from *nax* and wt cerebellum at P2 and P7; the Cdh4 mRNA differences between P2 and P7 are insignificant in wt PCs and wt BGCs; In *nax* PCs and BGCs, the Cdh4 mRNA increases by time; At P2 the values significantly rises (P <.05) in *nax* PCs and *nax* BGCs. **B)** qRT-PCR of total tissue between shows decreasing trend from P2 and P7 in both wt cerebellum and *nax* cerebellum. This experiment was repeated over three different litters for each postnatal day in wt and *nax* siblings (wt; n=3 and *nax*; n=3). The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using multiple comparison ANOVA. (P-value ≤.05 was considered as statistically significant.) Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells**.**

3.2.3 Cdh8 expression pattern analysis

Cdh8 is the only member of classic cadherin type II in our study that is expressed in brain, specially in PCs (94). To demonstrate the expression pattern of Cdh8 in cerebellar cortex, brain sections of both strains were double stained with anti-cd8 antibody and anti-CALB1. The cerebellum samples were collected from P5 and P7 as closest time points to the PCs excessive migration in *nax* strain. The expression of Cdh8 from IHC was stronger on the location of PCs (Fig. 7A, C) in wt cerebellum than the *nax* counterparts at P5 (Fig. 7B) and P7 (Fig. 7D), but the patterns were not clear enough for performing densitometry analysis. Following IHC, Western blot was performed to measure and compare whole cerebellum expression of Cdh8 in both strains at P2, P5 and P7. The expression pattern of Cdh4 from P2 to P7 is unchanged in wt cerebellum and increased in

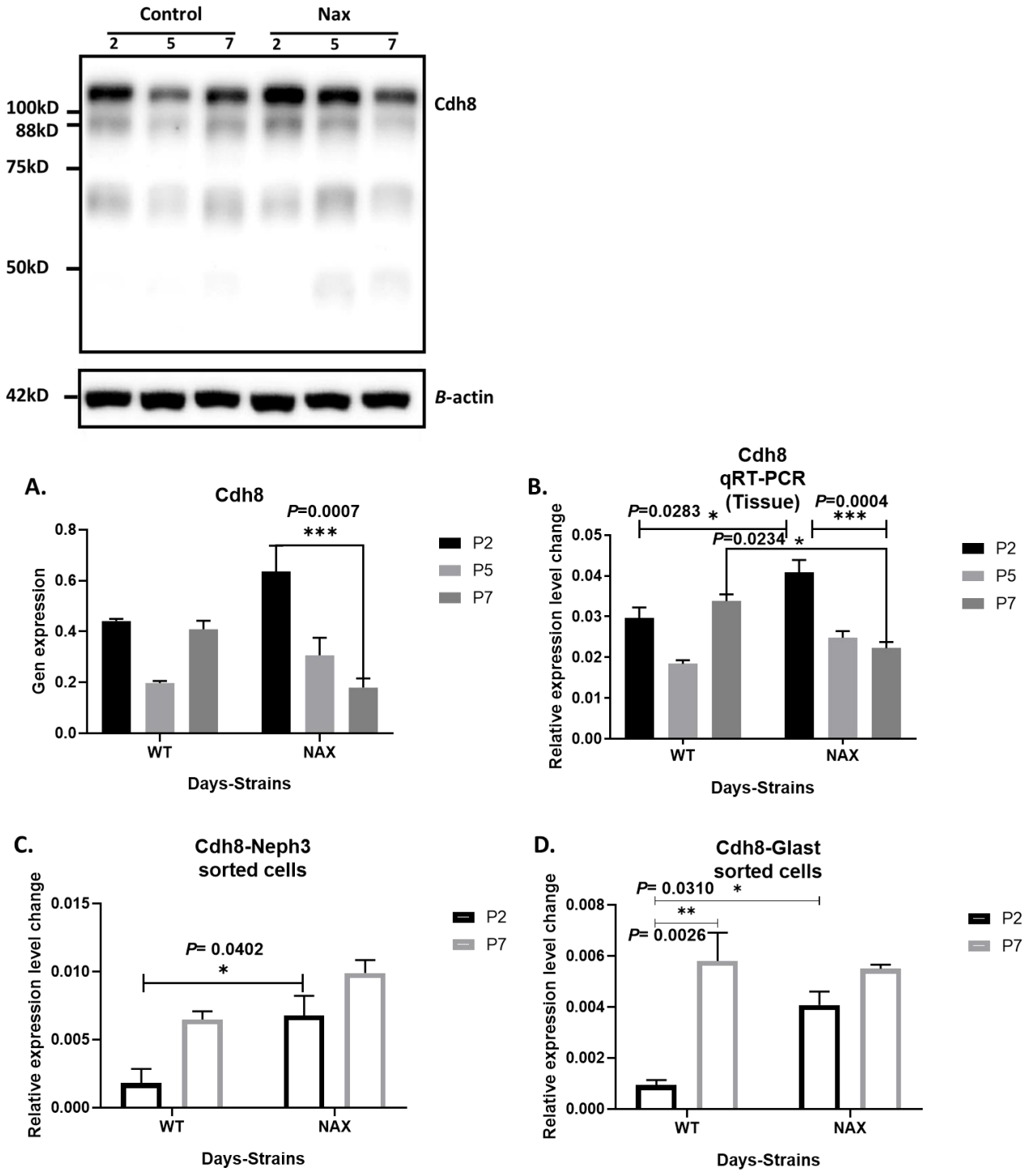
corresponding *nax* (Fig. 8A). The changing expression pattern in *nax* cerebellum at P2 also is in inverse proportion to the values at P5 meaning that the Cdh8 protein has to be upregulated/express more than an average at the beginning of coupled migration and be downregulated/express less than an average by the time both cells reach pcl. To confirm our findings at cell level, qRT-PCR was performed and measured the cellular (in PCs and BGCs) transcription of Cdh8 in cerebellum strains (Fig. 8C, D). The mRNA values in PCs and BGCs of wt and *nax* cerebellum are overall increased by time, although the values did not reach statistical significance (except wt BGCs at P7 P=0.0026.) Comparing cellular Cdh8 mRNA values between strains, mutation at P2 significantly increased transcription in both PCs and BGCs. At P7, however, PCs are the only cell type with different Cdh8 regulation in wt cerebellum from *nax* counterpart; the high mRNA level of Cdh8 at P7 coincides with positioning PCs in ML and therefore may play important role in PCs migration. To confirm our cell based qRT-PCR analysis, total Cdh8 mRNA was also quantified by qRT-PCR. mRNA result from the whole cerebellum shared a similar pattern to the total protein trend from P2 to P7 whether in wt or *nax* cerebellum (Fig.8B). Both (total mRNA and protein) analyses alike present higher values in *nax* cerebellum at P2 and lower at P7 when compared to wt counterparts. The mRNA-protein values are also downregulated from P2 to P7 in *nax* cerebellum which is contrary to increasing pattern in *nax* PCs/BGCs. The negative trend indicates an increasing transcription of Cdh8 in *nax* targeted cells while its protein expression across the *nax* cerebellum was downregulated.

Figure. 7. Colocation of PCs and Cdh8 in wt and *nax* cerebellar cortex



(A, C) Double immunostaining of wild type mouse sagittal sections with anti- Cdh8 (green) and anti-CALB1 (red) shows the strong immunofluorescent signals of Cdh8 (arrowhead) collocated with the cluster of PCs (arrow) at P5 and the monolayer of PCs in pcl at P7. **(B-D)** Double immunostaining with anti-Cdh8 (green) and anti-CALB1 (red) in sagittal sections of *nax* mouse cerebellum shows the Cdh8 expression in the cluster of PCs extended from pcl to pia surface at both P5 and P7. **(a-d)** Higher magnification of the local Cdh2 expression. Abbreviations: PCs=Purkinje cells. Scale bars: A = 50 μm (applies to panels **A, B, C and D**).

Figure. 8. Upregulation of Cdh8 in PCs and BGCs after mutation



A) Cdh8 expression in cerebellum is measured by western blot at P2, P5 and P7; protein expression in wt cerebellum is at he same level between P2 and P7; In *nax* cerebellum Cdh8 expression is

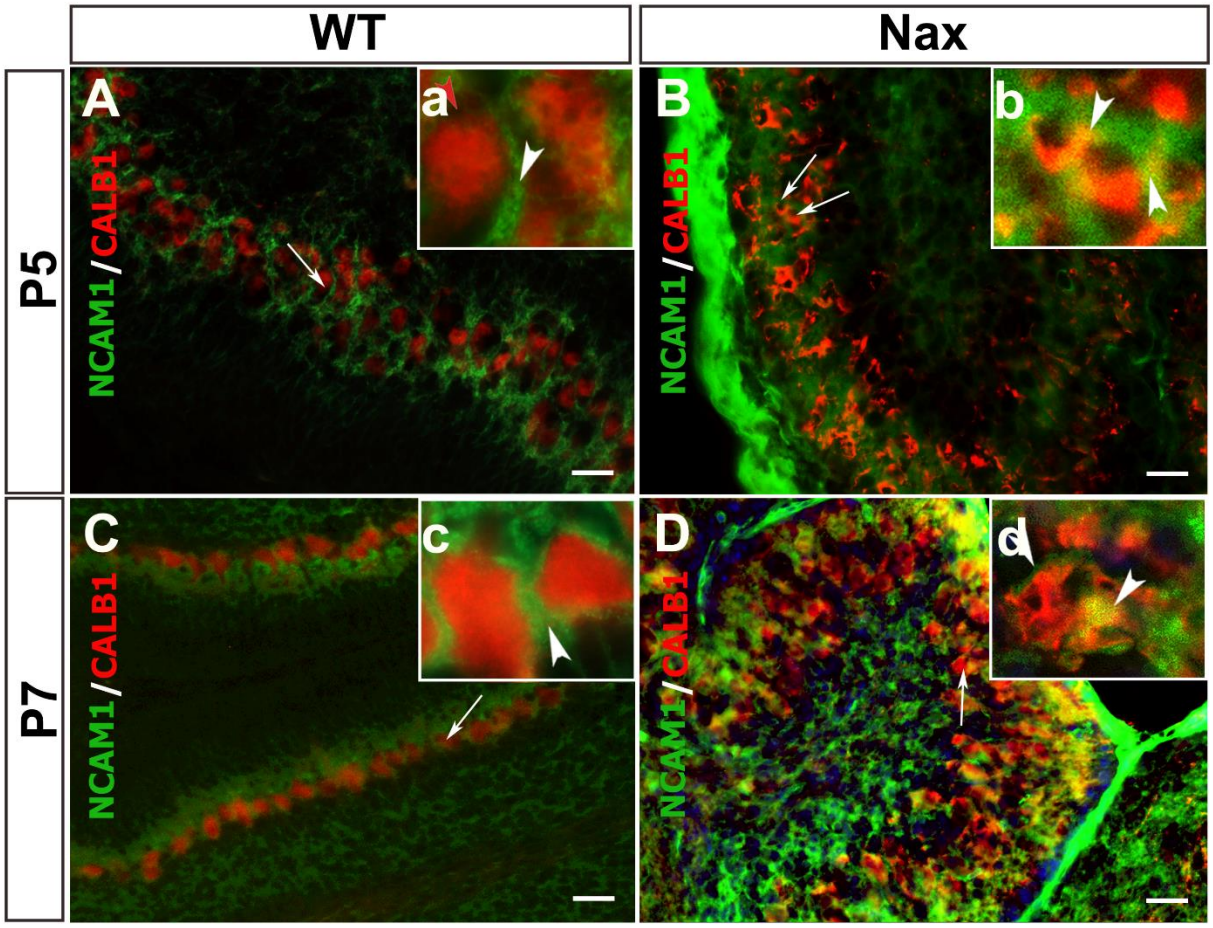
significantly decreased between P2 and P7 (*P<.001*), The values in *nax* cerebellum are higher at P2 and lower at P7 than wt cerebellum **C, D)** qRT-PCR of mRNA collected from isolated PCs and BGCs from *nax* and wt cerebellum at P2 and P7; the Cdh8 transcription in PCs and BGCs is increased between P2 and P7, but the differences only in wt BGCs reaches statistical significance (*P<.001*) **B)** qRT-PCR of total tissue between P2 and P7 shows a same level mRNA in wt cerebellum and decreased pattern in *nax* cerebellum. This experiment was repeated over three different litters for each postnatal day in wt and *nax* siblings (wt; n=3 and *nax*; n=3). The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using multiple comparison ANOVA (P-value ≤.05 and lower were considered as statistically significant.) Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells. Scale bars: A = 50 μm (applies to panels **A, B, C and D**).

3.2.4 Ncam1 expression pattern analysis

Neural cell adhesion molecule (NCAM) is a member of IgCAM superfamily broadly expressed in neuroglia network of CNS not only during embryonic days but also in adulthood (69, 111, 112). In cerebellum, the colocalization of the Ncam1 and PCs is demonstrated by double staining of cerebellar sections using anti-NCAM and CAlB1 antibodies respectively. The expression of Ncam1 in wt cerebellum showed strong signals around PCs at P5 (Fig. 9A) and P7 (Fig. 9C). Signals at P7 are expanded from the location of PCs toward pia surface and surround the PCs dendrites. In *nax* cerebellum, Ncam1 is expressed among the multilayer organizations of PCs at P5 (Fig. 9B). At P7 (Fig. 9D), Ncam1 is abundant around PCs from pcl location to the areas close to the pia surface. The Ncam1 fluorescent signals in *nax* cerebellum are week and less organized than the corresponding wt. Measuring density of signal due to the cerebellum structural

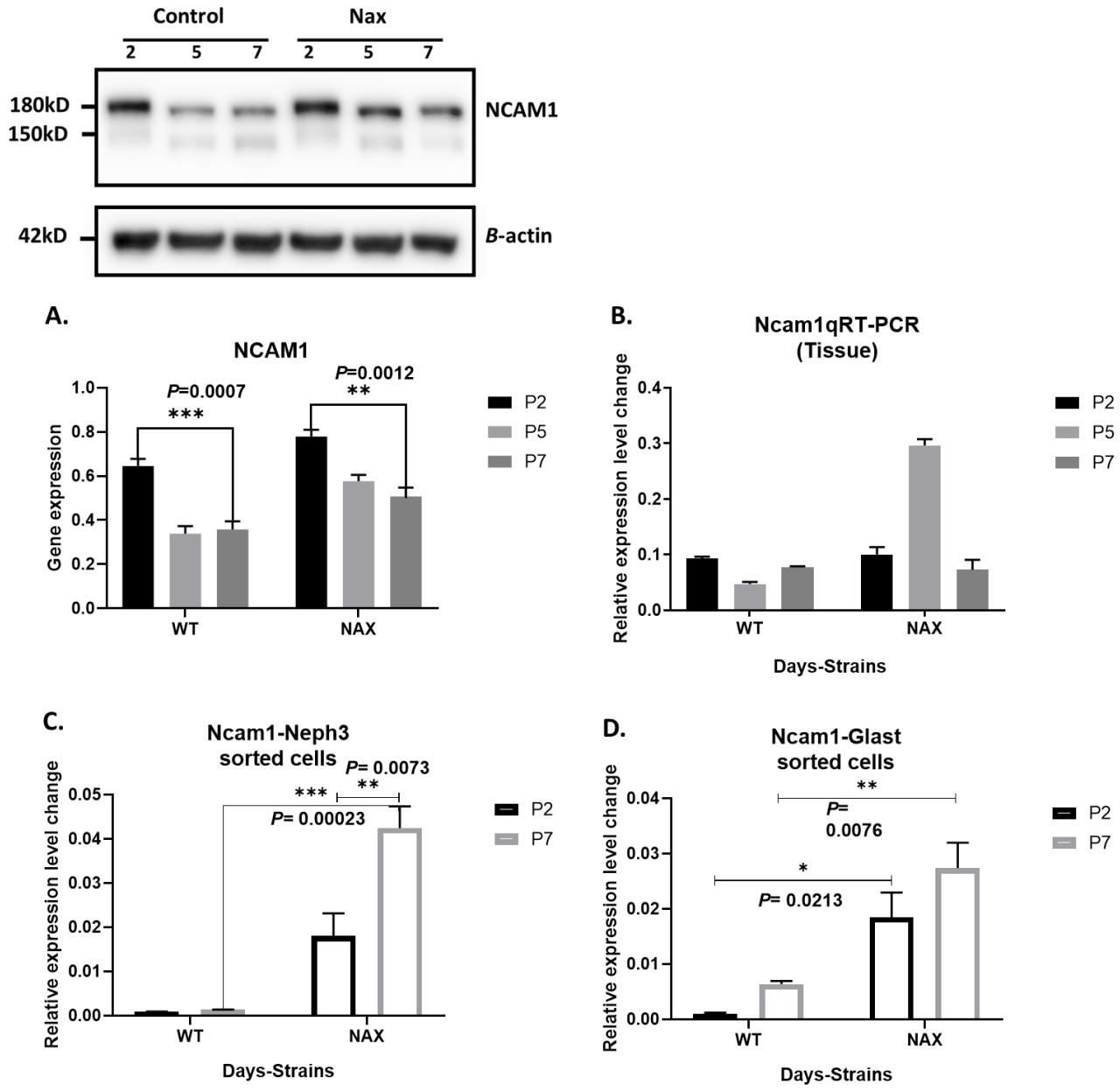
malformation would be inaccurate. Therefore, western blot was carried out using anti-NCAM antibody to measure the total expression of Ncam1 in *nax* and wt cerebellum at P2, P5 and P7. The expression of Ncam1 protein in both *nax* and wt cerebellum is decreased by P7 ($P<0.001$). The total Ncam1 expression is overexpressed in *nax* cerebellum, but the *nax*-wt value differences were insignificant (Fig. 10A). To understand how the collected protein data would correlate to the targeted cells, it was necessary to analyze the cellular expression of Ncam1 as well. Therefore, PCs and BGCs were isolated, their mRNA was extracted and analyzed by qRT-PCR at P2 and P7. Results showed that Ncam1 transcription in both PCs and BGCs are substantially enhanced by *nax* mutation. The expression is also increasing by time in both cells (PCs between P2 and P7; $P<0.001$) (Fig. 10C, D). In order to indirectly estimate the protein level of PCs in both strains, total Ncam1 mRNA of cerebellum was analyzed with qRT-PCR as well (Fig. 10B) and the results were compared to the collected Ncam1 data. Although the total mRNA result did not reach statistical significance, their pattern similar to the protein expression counterpart showed a downward trend by P7. Therefore, the only considerable change in total expression (mRNA/protein) of Ncam1 is between days not strains. The huge gap of Ncam1 expression in PCs/BGCs between *nax* and wt cerebellum which is leveraged at total protein and mRNA level confirms our cellular data with higher degrees of accuracy.

Figure. 9. Colocation of PCs and Ncam1 in wt and *nax* cerebellar cortex



(A, C) Double immunostaining of wild type mouse sagittal sections with anti-Ncam1 (green) and anti-CALB1 (red) shows the immunofluorescent signals of Cdh8 (arrow) intercellular (not overlapped with) of the PCs (arrowhead) in the cluster stage at P5 and in pcl at P7. **(B-D)** Double immunostaining with anti- Ncam1 (green) and anti-CALB1 (red) in sagittal sections of *nax* mouse cerebellum shows the Ncam1 expression in the cluster of PCs extended from pcl to pia surface at both P5 and P7. Immunofluorescence staining for Ncam1 (green) shows strong immunoreactivity in pia surface of the *nax* cerebellum at P5 and P7. The expression of Ncam1 overlaps with the PCs soma at both P5 and P7 **(a-d)** Higher magnification of the local Cdh2 expression. Abbreviations: PCs=Purkinje cells. Scale bars: A = 50 μm (applies to panels **A, B, C and D**).

Figure. 10. High Ncam1 transcription in *nax* PCs and BGCs, but not in whole cerebellum



A) Ncam1 expression in cerebellum is measured by western blot at P2, P5 and P7; protein expression in wt and *nax* cerebellum is significantly downregulated between P2 and P7 (wt cerebellum (*P<.001*) and *nax* cerebellum (*P<.05*)). **C, D)** qRT-PCR of mRNA collected from isolated PCs and BGCs from *nax* and wt cerebellum at P2 and P7; The Ncam1 mRNA in PCs and BGCs are significantly higher in *nax* cerebellum than the wt counterparts (PC at P7: *P<.001*, BGCs

at P2 and P7: $P<.05$); The differential expression between P2 and P7 are insignificant in *nax* PCs and *nax* BGCs. **B)** qRT-PCR of total mRNA did not show any variation in wt PCs and wt BGCs between P2 and P7 and the values are similar to the corresponding *nax*. This experiment was repeated using three different litters for each postnatal day in wt and *nax* siblings (wt; n=3 and *nax*; n=3). The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using multiple comparison ANOVA (P-value ≤.05 and lower were considered as statistically significant.) Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells. Scale bars: A = 50 μm (applies to panels **A, B, C and D**)

CHAPTER 4: DISCUSSION

Cerebellum has a critical role in brain function. Cerebellar cortex is composed of three layers. The PCs in the pcl layer are the only output of the cerebellar cortex. Damaging and loss of these cells in traumas and neurodevelopmental and neurodegenerative diseases such as ASD (7, 8) and ataxia (9-12) have been frequently reported in human. In respect to treatment, therefore, understanding developmental process and physiological behaviours of neurons is necessary to generate a new neuron, place them in the correct location and maintain their functions afterward. Most of the studies so far have been focused on reprogramming stem cells to new PCs (in mouse) while our knowledge on their migration particularly in post-natal mouse cerebellum is limited (19). The precursor of mouse PCs emerges between E10.5 and E13 and begins to migrate at around El2 just after the final mitosis (130). Several studies suggested that during embryonic days in mouse about E12 the radial glia fibre from ventricular zone servs as a guidance for migration of PCs as they ascend in a multilayer conformation (cluster) toward the pia surface (130, 131). PCs were also suggested to follow a tangential route parallel to the nascent cerebellar surface and about perpendicular to the orientation of nascent radial glial fibres during embryonic days (132). The interdependency of PCs and BGCs for differentiation of each other from embryonic days (precursors of both cell types) to the late postnatal days have been frequently reported (133, 134). Movement of PCs is probably guided by BGC fibres mediated by cell adhesion molecules from embryonic days to the early postnatal (E13 to P0) (130). Therefore, there are several evidences supporting the close relationship between PCs and BGCs. However, the presence of BGCs at early stage of cerebellar development in not clear (135) and it also has not been discussed whether the migration of PCs dependant on their attachment to glia cell body (somite) during postnatal days (130). This is one of the main reasons of investigating postnatal PCs in our experiment. I realized

that the movement of PCs from postnatal day 2 is accompanied in the same direction with BGs (soma) which are just emerged from radial glia precursors. The coupled movement of these cells were confirmed in not only normal cerebellum but also in a mutant cerebellum such as *Reeler* pathway mutant with ectopic PCs and in *nax* with excessive PC migration in molecular layer (25). Therefore, **I hypothesize that Bergmann glia cells (BGCs) play a crucial role for PCs postnatal dispersal and monolayer formation**. In order to understand how physical interactions between neuron, glia and ECM would impact the PCs-BGCs coupled migration and what type of the cell membrane proteins are involved, a group of promising cell adhesion molecules (CAMs) were selected and their expression pattern in cells and tissues were compared between *nax* and wt cerebellum.

The selected CAMs in our study are members of classic cadherin type I, classic cadherin type II and immunoglobulin superfamily CAMs (IgCAMs), which are associated in at least one of the coupled migrated cells that have been confirmed in previous studies. The type I classic cadherin (e.g. Cdh2, Cdh4) preferably mediate homophilic connection between two cells which means both cells have to produce and share the same cadherin to interact with each other. The dimerized cadherins are semi-strong, but their concentration at the interfacial space strengthens the cell-cell connections. The type II classics cadherin (Cdh8) structure is similar to type I, but little differences are to their advantage to not only mediate homophilic connections but also interact with different type of CAM in a heterophilic bond. The Ncam1 similarly integrate cells in homophilic and heterophilic manner as well. Their interaction with matrix compounds such as integrin greatly impacts the cell-ECM connections in respect to increasing cell motility. Therefore, data about changing the expression of these CAMS would give an idea about the status of cells during migration.

4.1 Ncam1 expression during Purkinje Cell Monolayer formation

Ncam1 expression in wt PCs and wt BGCs

NCAM is the only member of the analyzed CAMs which is not categorized in classic cadherin superfamily. Their expression in CNS promotes neural cell migration, neurogenesis and differentiation (115, 123, 136). NCAM mediates homophilic and heterophilic cell-cell and cell-ECM adhesions (137). In this study, it seems that the expression of Ncam1 is greatly affected by ACP2 mutation. Ncam1 is barely expressed in PCs and BGCs of wt cerebellum at P2 which may indicate that Ncam1 is not actively involved during the time in cell coupled migration. This may be reasonable as the post natal coupled migration is at its very early stage and it is expected to see some changes in Ncam1 expression as both cells are migrating further from pcl. However, only wt BGCs shows increasing expression of Ncam1 at P7. Since BGCs are the carrier of PCs in my hypothesis, observing the aforementioned pattern only in these cells is not far from expectations. Ncam1 is reported to regulate the directional lamellipodia formation and migration of bone marrow-derived mesenchymal stem cells (BMSCs) via b1 integrin signalling. The blocking of b1 integrin in Ncam1 KO BMSCs rescued the migration of these cells (138). Studies also showed that the cleavage of NCAM140 (one of the three NCAM isoforms) in B35 neuroblastoma cells promotes the integrin dependent migration of these cells to extracellular matrix proteins (139). Therefore, these studies indicated 1) the potential inhibitory effect of integrins on Ncam1 expression and 2) the decreasing NCAM -such as NCAM140 isoform- in cells motivates their integrin mediated migration. Therefore, it seems that the inhibitory effect of integrin on Ncam1 expression in general potentiates cell migration. The result from my study suggest that the expression of Ncam1 in wt PCs and wt BGCs were similarly limited and replaced by the function of integrins to promote migration of these cells. I speculate that by P7, the integrin-Ncam1

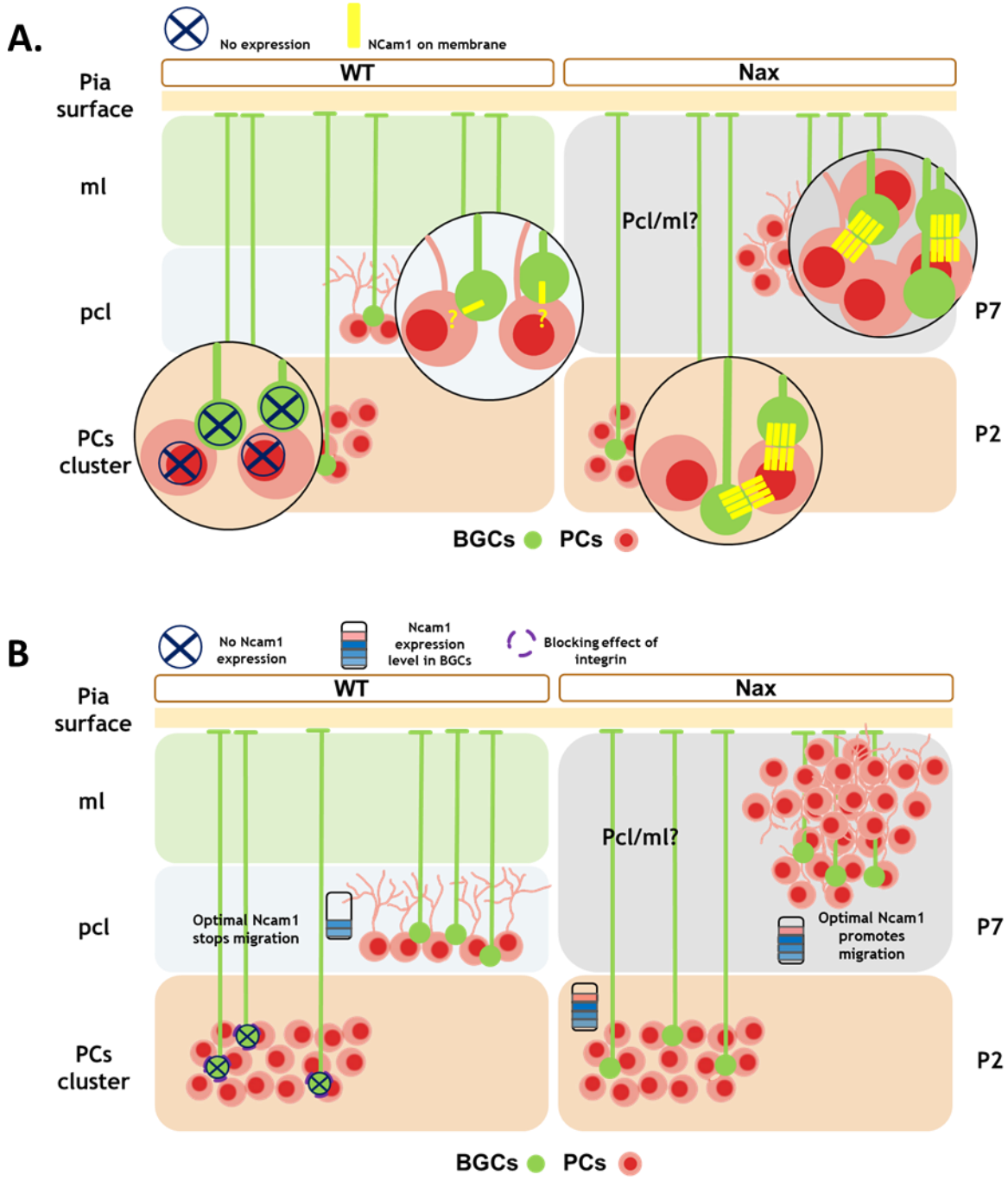
regulation changes to increase Ncam1 at pcl and stop the BGCs' migration. This process if mediated by Ncam1 may need an optimal level of Ncam1 expression as well to warrant cell migration. Other in vitro and in vivo studies also showed the effect of Ncam1 expression on reducing the migration of glioma cells (140, 141) and this implies that Ncam1 expression in BGCs may potentiate migration inhibition in these cells too. Therefore, I speculate that inhibition of Ncam1 expression during early postnatal days potentiates wt BGCs migration. However, rising expression of the same molecule later at P7 stops wt BGCs in pcl.

Ncam1 expression in nax PCs and nax BGCs

Is it possible that overexpressing the same molecule (Ncam1) regain the migration of BGCs which are expected to be stopped at pcl? I speculate that the BGCs at pcl can return to Ncam1 mediated migration if the specific range of Ncam1 expression is supplied in these cells; Providing the sufficient Ncam1 for returning to migration is speculated to not be fulfilled in wt BGCs, but the corresponding *nax* cells. In this regard, reports showed that NCAM protein activates the migration and neurite growth in Schwann cells by mediating signalling pathways as a coreceptor of the glial cell line derived neurotrophic factor (GDNF) receptor GFRa1. The GDNF signalling mediated by NCAM finally decreases the NCAM-dependant adhesion which inhibits the cell movement. This indicates that the optimal level of cell-substratum (mediated by NCAM) is required to warrant the motility of the cells (142). The overexpression of Ncam1 in several metastatic cancers such as neuroblastoma, small cell lung carcinoma, renal cell carcinomas, and Wilms' tumor may similarly contribute to providing the Ncam1 mediated optimal cell-stratum interaction (143). Therefore, my study may indicate that the overexpression of Ncam1 in *nax* BGCs provides an optimal cell-stratum interaction and promotes the excessive migration of these cells in cerebellar cortex. It has been already established that the NCAM interacts with β1 integrins modulates adhesion to

extracellular matrix proteins (ECM) (142). The ECM in cerebellar cortex is mainly composed of laminin and collagen which are both recognised by β1 integrins (144). Therefore, the regulation of BGCs migration in both strains my proceed through the Ncam1-integrin interactions. Result may also suggest that Ncam1 is less likely to directly interconnect BGCs and PCs during early postnatal days or excessive migration. Ncam1 has high affinity to establish homophilic connections which requires offering Ncam1 by both cells. Results shows that Ncam1 is expressed in neither of wt PCs and wt BGCs at P2. Even at P7, only wt PCs express Ncam1 which is not enough for establishing a Ncam1 mediated connection. Comparing these results to the Ncam1 overexpression in *nax* cells (PCs and BGCs) is not reasonable as the ultimate goal of using mutant cerebellum is to understand the function of Ncam1 in wt cerebellum. Therefore, the results of this study may suggest that Ncam1 is less likely to be involved in connecting BGCs and PCs (Fig. 11A), and instead it may regulate the migration of BGCs particularly at P7 when these cells are expected to either stop or continue migration (Fig. 11B).

Figure. 11. Ncam1 only regulates the excessive migration of BGCs



A schematic illustration of cerebellar development at sagittal section of postnatal mice. **A)** shows that despite the overexpression of Ncam1 in *nax* PCs and *nax* BGCs, this molecule may not mediate BGCs-PCs connection at P2 and P7. **B)** There is a possible integrin-Ncam1 relationship

in BGCs that promotes their migration at P2 and stops them at P7. Overexpression of Ncam1 in *nax* BGCs may provide an optimal cell-stratum interaction and reactivate the migration in the settled BGCs at pcl. Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells; pcl=Purkinje cell layer; ML= Molecular layer; pcl/ml(?): There is no border between pcl and ml.

4.2 Cdh4 expression during Purkinje Cell Monolayer formation

Cdh4 expression in wt PCs and wt BGCs

Cdh4 is a member of classic cadherin type I which by expression in subtypes of glia cells plays a critical role for axonal guidance in optic nerve (85). As a classic cadherin, Cdh4 is expected to have high affinity for trans-homodimers establishment between cells (145), meaning that the same molecule is shared between the adjacent cells. Unequal presentation of Cdh4 by either of integrated cells could potentially loosen the PCs-BGCs connections. My results showed that the Cdh4 mRNA rises in wt PCs while it is unchanged in BGCs. Although this pattern does not meet the criteria of Cdh4 mediated connection (interconnected cells ideally share the same amount of Cdh4 in the interfacial space), both PCs and BGCs still manage to reach and settle at pcl by P7. Explaining how this pattern is influenced by Cdh4 expression may rely on understanding the interaction of this molecule with other molecules. In this regard, several reports established involvement of Cdh4 in cell-cell and cell-ECM interactions (146) which leads us to three different speculations about how the expression pattern of Cdh4 in wt PCs and wt BGCs could contribute to their behaviors at P2 and P7: 1) The results of our study may initially suggest that Cdh4 does not play a critical role in establishing connections between wt PCs and wt BGCs as only one cell (PCs) integrates rising Cdh4 between P2 and P7 which does not suit a homophilic interaction; 2) The capacity of Cdh4 to mediate heterophilic connections leads to an alternative explanation that finds the time dependant

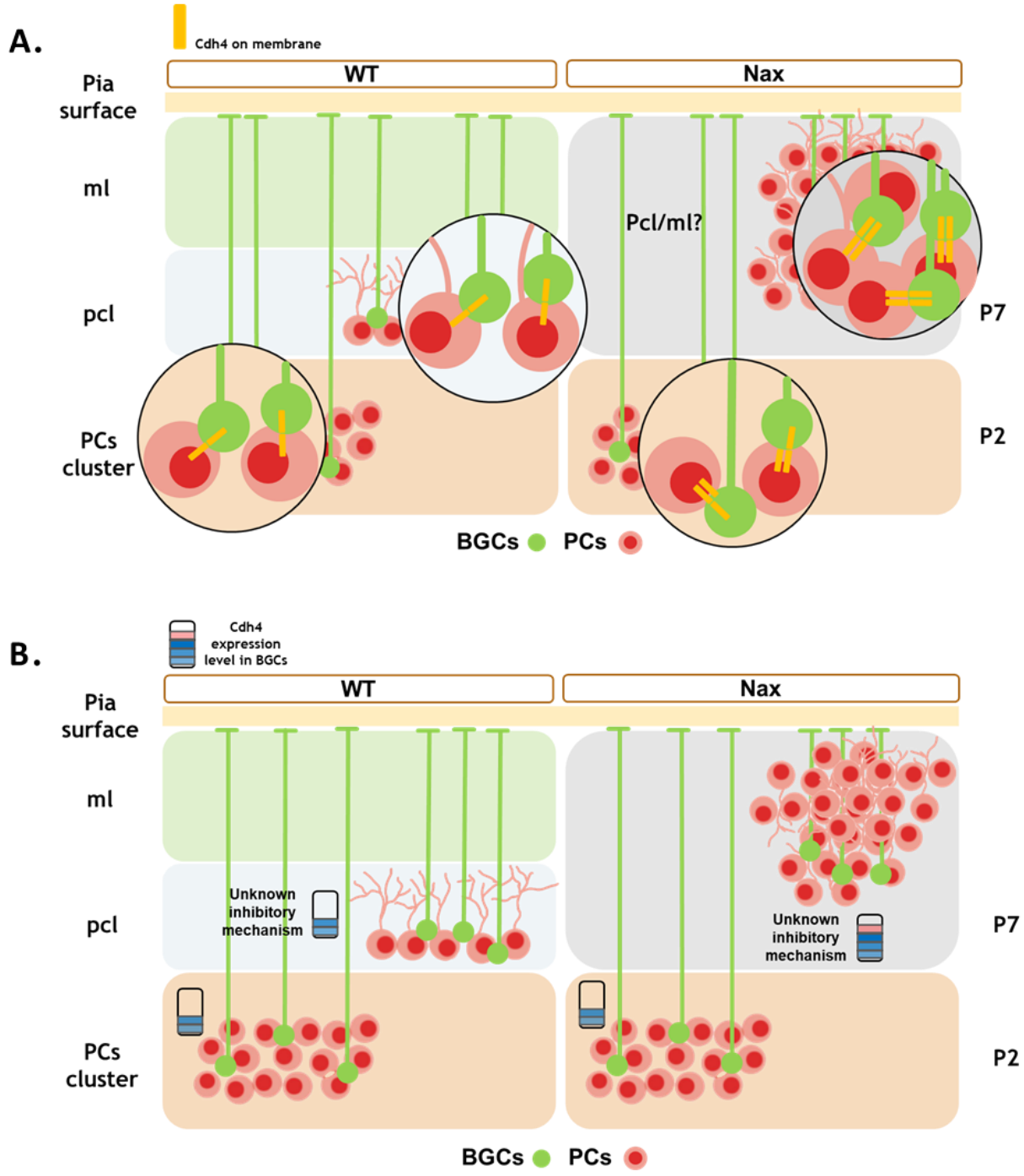
upregulation of Cdh4 in wt PCs as a compensatory mechanism for lack of the same molecule in wt BGCs. The extra Cdh4 in wt PCs may interact with other adhesive molecules in wt BGC and recover the Cdh4 mediated connection between two cells; 3) The last explanation particularly about the expression of Cdh4 in BGCs, which is unchanged between P2 and P7, may suggest that the motility of BGCs requires specific level of Chd4 expression, which if changes may disrupt the cell migration. The two latter speculations fulfill the prerequisites of the BGCs-PCs coupled migration which are establishing a strong neuroglia connections and motility of the carrier cell (BGCs) in wt cerebellum.

Cdh4 expression in nax PCs and nax BGCs

The Cdh4 overexpression in *nax* PCs and *nax* BGCs at P7 may imply the role of this cadherin in maintaining BGCs-PCs attachment for a longer distance from pcl. By the time PCs and BGCs are located in pcl, it is unclear what type of regulation or cell-ECM interactions related to Cdh4 would stop BGCs in pcl. Result shows that the Cdh4 expression is simultaneously exceled in PCs and BGCs at P7 after mutation which coincides with further migration of both cells toward pia surface. I speculate that the unknown migration inhibitory mechanism in pcl is overcome by the overexpression of Cdh4 in *nax* BGCs, which as a result promotes the excessive migration of these cells in cerebellar cortex. The excessive migration of *nax* BGCs may occur using soma translocation followed by the Cdh4 regulated integrin-ECM interaction in these cells. It is not clear that if BGC soma in particular is using soma translocation from the beginning of coupled migration. However, studies showed that lack of certain ECM components in mouse cerebellum such as Laminin α1 adversely affects organization of BGCs (147, 148). Other studies even showed that some ECM members such as tenascin, astroglia derived ECM, assigns expression pattern to the BGCs locations across the cerebellum (131). Therefore, the interaction with ECM is an

important factor for normal migration of BGCs and the sudden upregulation of Cdh4 at P7 could potentially activate this feature to serve soma translocation in *nax* BGCs. Therefore, results may suggest that Cdh4 not only mediates intercellular connection between PCs and BGCs (Fig. 12A) but also promotes the migration of BGCs, which are attached to PCs, particularly during excessive migration (Fig. 12B).

Figure. 12. Cdh4 mediates BGCs-PCs connection and regulates the coupled migration



A schematic illustration of cerebellar development at sagittal section of postnatal mice. **A)** Cdh4 may mediate the BGCs-PCs connection in wt and *nax* cerebellum, but their interconnecting role

during excessive migration becomes more important as Cdh4 is overexpressed in both cells, unlike the corresponding wt cells, and potentially establishes stronger connection between cells. **B)** Cdh4 especially in BGCs is expressed similarly in both strains at P2. At P7, there are presumably unknown migration inhibitors. The expression of Cdh4 in BGCs is not enough to overcome the inhibition and cells stop at pcl. The overexpression of Cdh4 in the corresponding *nax* BGCs may overcome the inhibition and promote migration. Abbreviations: PCs=Purkinje cells; BGCs=Bergman glia cells; pcl=Purkinje cell layer; ML= Molecular layer; pcl/ml(?): There is no border between pcl and ml.

Comparing patterns of Ncam1 and Cdh4

Both Ncam1 and Cdh4 share a downward total mRNA-protein expression trend between P2 and P7 in both strains. The fact that this trend in *nax* cerebellum is similar to the corresponding wt may shed light on an underlying regulatory mechanism so restricted to allow any effective change in the pattern of these two genes after the ACP2 mutation. Moreover, the cellular expression of Ncam1 and Cdh4 in PCs and BGCs is negatively associated to the total (mRNA-protein) pattern over the time. This relationship may imply that the majority of the total mRNA-protein evaluated from *nax* and wt cerebellum is expressed in a few types of cells like BGCs and PCs.

4.3 Cdh2 expression during Purkinje Cell Monolayer formation

Cdh2 expression in wt PCs and wt BGCs

Cdh2 is a typical classic cadherin type I which only mediate homophilic connections (149, 150). This means each Cdh2 molecule on PCs should dimerize with the same type of molecule on the

adjacent BGCs. This feature of Cdh2 justifies the upregulation of Cdh2 in both types of cells from wt cerebellum over the time may indicate developing intercellular connections along the migration.

Cdh2 expression in nax PCs and nax BGCs

The ACP2 mutation on the other hand created a pattern where Cdh2 is overall overexpressed in *nax* PCs, but does not change in *nax* BGCs at P7. The same level of Cdh2 expression in BGCs from both strains coincides with their different locations in cerebellar cortex at the same time (P7). The *nax* BGCs are excessively migrated from pcl while the corresponding wt stop at pcl. One explanation for this pattern is that the function of Cdh2 in BGCs is probably affected by the ECM stimulants that are different in *nax* from wt cerebellum. The effect of ECM on BGCs migration has been already established (131, 151). These interactions may be partially mediated by Cdh2 molecules. Studies showed that sequential interaction between integrin-ECM and Cdh2 may promote the motility of the cells (152). In 2011, a model of ECM-integrin-Cdh2 interaction was proposed where the effect of fibronectin (ECM protein) assembly near mouse embryonic fibroblasts (MEF) cells translocate the inactive α5β1 integrins at the adjoining location to the part of the membrane where cell interacts with ECM and this may activate α5β1 integrin and promote integrin-dependent cell migration (152). This model may apply to BGCs, but the main question is that how the similar expression of Cdh2 between *nax* and wt BGCs could impact the BGCs-ECM interaction that lead to different migratory behaviors in these cells. I believe that the answer is embedded in activation of Cdh2 mediated regulations being partially dependant on extracellular stimulants. Studies showed that time and type of the stimulation defines the following signalling pathway in a cell and even give rise to different outcomes from a single pathway (153). We have not yet profiled these molecules in *nax* cerebellum, but I believe that changing Cdh2 expression particularly at pcl (P7) could be one of the reasons of excessive migration in *nax* BGCs. Changing

extracellular stimulants in pcl may trigger different inputs in the same cell even if Cdh2 expression doesn't change (wt BGCs and *nax* BGCs at P7) and the outcome may impact the cell migration. Other studies also showed that the active cascades of intracellular signals (signal of migration in our case) can be effective over large scale of time. However, starting the signal may require an optimal level of cell ECM interaction (154). This means that cell may need to express and transfer specific number of CAMs on the membrane to interact with integrin and ECM. In my results, the minimum level of Cdh2 expression at P2 is believed to be the activator of a signaling cascades that persist for next 5 days. By P7, the pathway may trigger the settlement of wt BGCs. However, in *nax* BGCs, the necessary signaling cascades are not even started because the expression of Cdh2 is more than usual in these cells. Moreover, by maintaining the same level of Cdh2 by P7, the *nax* BGCs are still prone to excessive migration. Therefore, *nax* BGCs may use Cdh2 mediated migration, but cannot stop by Cdh2 mediated signaling. Therefore, our result may show that the overexpression of Cdh2 at P2 does not disrupt the migration of BGCs, but it decreases the chance of the cells to start/remain active signaling cascades that at P7 can stop BGCs. In the same study conducted by Lefort et al. in 2011 (152) the integrin-fibronectin interaction also decreased the Cdh2-Ctnnb1 complex (not downregulation of Cdh2) in the adjoining location and as a result the Cdh2 mediated cell-cell connection becomes weak. Lack of the enough Cdh2 active form may disrupts the BGCs-PCs connection, but I believe that there is a capacity of reviving neuroglia connection by compensatory mechanisms that involve Cdh4 expression in BGCs. In this regard, our results showed that changing expression of Cdh2 between *nax* and wt BGCs at P7 is in an inversed proportion to the corresponding patterns of Cdh4 in the same cell. This may indicate a mutual beneficial relationship between Cdh2 and Cdh4, where the Cdh2 loss/inhibition in *nax* BGCs might be compensated by increasing Cdh4 in the same cell. Moreover, despite the high

affinity of Cdh2 to homophilic cell-cell interactions studies suggested that these molecules can also potentiate the establishment of cis heterodimer (heterophilic connection) with Cdh4 between two cells. Each cell offers only one type of the aforementioned cadherins in their adjoining cell surfaces. Therefore, Cdh2 is capable of mediating homophilic and heterophilic (exceptional cases) cell-cell connections. Accordingly, the overexpressed Cdh2 in *nax* PCs may interact with Cdh2 alternatives such Cdh4 on BGCs to save the neuroglia connections. Therefore, it is less likely that the ACP2 mutation could severely affect the Cdh2 mediated connections between BGCs and PCs whether at P2 or at P7, during excessive migration. Besides the cell-cell connection, Cdh2 is suggested to be involved in regulation of cell velocity (speed) and direction as well (155). In line with the same idea, I speculate that the different cellular localizations and Cdh2 expression in BGCs between two strains are indicative of controlling the velocity to promote excessive migration of these cells in *nax* cerebellum. Firstly, the cluster of PCs is extended from pcl to pia surface. The further location of some PCs may imply increasing migration speed in fraction of *nax* PCs population. I believe that this is partially due to changing local stimulants in *nax* cerebellum. It may also be due to the activation/inactivation of pathways that were only triggered by low Cdh2 expression in wt BGCs at P2 and the overexpressed Cdh2 in *nax* BGCs may have changed the parameters for normal speed migration. Therefore, it is unlikely for the Cdh2 expression to peak at P7 in BGCs and suddenly activate their cell migration. However, this process is very complicating and yet to be proved by monitoring the spatiotemporal dynamic of BGCs and PCs. Overall, Cdh2 may regulate the migration of BGCs in normal cerebellum and play a regulatory role in stopping or promoting their migration at pcl. It is suggested that Cdh2 is one of the main mediators of intercellular connection for BGCs-PCs coupled migration.

4.4 Cdh8 expression during Purkinje Cell Monolayer formation

Cdh8 expression in nax and wt BGCs

Cdh8 is a member of classic cadherin type II which can establish homophilic cell-cell contacts and their extracellular domain specific sequences gives them more affinity to heterophilic connections than classic cadherin type I. By comparing the expression pattern of Cdh8 in PCs and BGCs, I realized that the differential expression between P2 and P7 is decreased after mutation. This pattern is very similar to the Cdh2 expression in BGCs (*nax* and wt) which was proposed to mediate cell motility through integrin-ECM interaction. The similar expression pattern of Cdh8 to Cdh2 particularly in BGCs and the general concept that Cadherins and integrins are involved in cell-cell and cell-ECM physical linkage may imply that Cdh8 also has the similar impact on directing BGCs migration as Cdh2. Therefore, based on the Cdh8 analysis in wt BGCs I speculate that the intracellular pathway that can target wt BGCs-residing at pcl may require the minimum level of Cdh8 expression provided at P2. The increasing Cdh8 expression after P2 can play a critical role in developing sequence of pathways that ultimately stop wt BGCs, while the overexpression of Cdh8 in *nax* BGCs at P2, which remains unchanged at P7, may disrupt this process. Therefore, since by the time the *nax* BGCs reaching pcl/ml location they are not prepared for residing in pcl, *nax* BGCs continue their migration (excessive migration). This may be the main reason that the similar expression of Cdh8 in BGCs between two strains at P7 leads to stopping the wt BGCs and excessive migration of the *nax* BGCs.

Cadh8 expression in nax and wt PCs

But how the expression of Cdh8 in PCs (*nax*/wt) may help the BGCs-PCs coupled migration. The expression of CDh8 in wt PCs similar to wt BGCs increases by time. The synchronized upregulation may implicate increasing the Cdh8 mediated neuroglia connection as both cells

become closer to the pcl. However, unlike in BGCs, the Cdh8 in *nax* PCs is over expressed at both P2 and P7. The over expression of Cdh8 in *nax* PCs at P2 is unlikely to impact the migration of these cells the way that I proposed in *nax* BGCs. The reason is that the BG soma plays a potential carrier, not PCs, and any change in Cdh8 expression that disqualifies BGCs from residing at pcl may be applied to the coupled PCs as well, but the otherwise is unlikely. Therefore, the overexpression of Cdh8 in *nax* PCs particularly at p7 may simply serve stronger connections with Cdh8 and other cadherins on *nax* BGCs. Additionally, the IHC images from sagittal sections of mutant cerebellum (Fig. 2F) showed that there is a multilayer conformation/cluster of PCs in *nax* in pcl/ml location, which indicates that not all *nax* PCs reside in the same excessive distance from pcl at P7. This may be partially because the gene regulations that set the migration pattern for PCs are not applied the same for the whole population. In this regard, the expression of Cdh4 and Cdh8 have been reported only in subset of PCs that formed stripes pattern. The stripes of Cdh4 and Cdh8 overlap each other only in certain areas (88). Although I did not show the local transcription of Cdh2, Cdh4 and Cdh8 in our experiment, the variation in *nax* PCs positioning (from pcl to pia surface) may be subjected to sub divisional expression of both Cdh4 and Cdh8 in one group of PCs. Therefore, it is important to consider both cadherins in the future for PCs analysis. Therefore, my study shows that the Cdh8 molecule, similar to Cdh2, may regulate the migration status of the BGCs at pcl. Cdh8, along with Cdh2 and Cdh4, may also involve in BGCs-PCs connection during the coupled migration.

CHAPTER 5: LIMITATIONS, FUTURE DIRECTIONS AND CONCLUSION

5.1 Limitations and future directions

Our experiment has potential limitations. The results although addressed the questions of my hypothesis, still are insufficient to provide comprehensive answers with higher degrees of

certainty. This study is the first FACS-based attempt to isolate PCs for qRT-PCR analysis and previous relevant studies are limited. Although all effort was put into optimizing the process, it seems that the increasing sample size to at least 5 per each time point would be more accurate and can confirm the reliability of our data. Moreover, the purpose of this study was to understand how CAMs are important for PCs and BGCs migration. My deductions/speculations based on CAM analysis at only the beginning and end of the coupled migration would not be able to provide a definitive answer and therefore in the future I may collect and analyze samples from random days between P2 and P7 to address the issue. In this study enhancing or inhibiting effect of transcription factors were not considered and the speculations were simply based on the difference between mRNA transcription pattern in cells and tissues. Moreover, high or low abundance of the mRNA does not necessary warrant the same pattern at protein level which means that profiling mRNA-protein expression in cells is a very complicated process requiring further studies. The PCs and BGCs were purified from the pool of cerebellar cells based on the cell specific surface markers. It was not possible to sort the PCs and BGCs based on the type of CAMs located on their membranes. Adding this information in the future would give us an idea about how many of each cell type is expressing specific marker at each time point. By purifying the sorted PCs and BGCs based on one type of the CAM molecules each time, the final qRT-PCR carried on the extracted mRNAs would provide more accurate results than in the method used. Due to the time limitation, the sole purpose of performing IHC in this study was to show the expression of CAMs in PCs and BGCs and not to explore or measure the expression patterns across the cerebellum. The ideal strategy would be distinctively visualized CAM expression in PCs and BGCs of mediolateral and anterior-posterior parts of cerebellum and measure the differences between time points and strains. I may perform the whole mounting and IHC analysis on central and lateral sections of cerebellum from

both strains. Since the location of CAM expression is not clear in IHC images, I decrease the thickness of the sections and perform densitometry analysis to estimate the differential expressions based on the emitted signals. The alternative option would be using a conditionally knocked down CAM model to assure that the visualized expression at each time point is coming from either BGs or PCs or both. Since the current knowledge about real-time migration of PCs-BGCs is very limited especially during P2-P7 postnatal days, the cerebellar sections from the transgenic mice can be cultured in vitro and the reaction of BGCs/PCs to low CAM be monitored during migration as well. Another experiment that can be designed to prove the BGCs-PCs coupled migration is to measure the pulling force of the fibres themselves. It is already known that there is tension between the terminals of the BGCs fibres and pia surface that correlates with the migration of BG cell bodies. Attachment or detachment of PCs to BGCs by promoting CAMs deficiency or PCs death can definitively change the tension in pia surface and therefore prove the offered PCs migratory mode. Part of the speculations from the results of this study was based on previous published results on CAM mediated Cell-ECM interactions in normal cerebellum. Moreover, the speculation of Cdh2 and Cdh8 starting long-term signalling is based on analysing signalling pathways other than those mediated by cadherins. Therefore, I believe that including further experiments in the future on profiling cerebellar ECM proteins particularly at pcl and targeting long-term signalling pathways regulated by cadherins would provide a more accurate insight into the BGCs-PCs coupled migration.

5.2 Conclusion

In this study I revealed the spatiotemporal distribution of CAM members in PCs and BGCs from the beginning and end of the coupled migration. My aim was to understand how these data would contribute to the different migration patterns of PCs and BGCs between nax and wt cerebellum

and ultimately suggest that the migration of PCs may be dependent on their attachment to BGC soma. In this study the expression and mRNA (cell and tissue) level of Cdh2, Cdh4, Cdh8 and Ncam1 were investigated in both strains. It is speculated that the characteristics of all four studied CAMs along with profile of their expression in BGCs strongly supports the possibility of BGCs-PCs connection at P2 and P7. However, the migration of BGCs as a carrier of PCs seems to be differently affected by the CAM members. Results shows that, the expression of Cdh2 and Cdh8 is strictly regulated in PCs and BGCs at P7. These regulations particularly in BGCs may start long-term signaling response from early postnatal days that only become effective (stop migration) when BGCs arrive at pcl and over expression of Cdh2 and Cdh8 may disrupt this process. The present findings may also confirm the critical role of Ncam1 and Cdh4 in regulating migration of BGCs at pcl. The overexpression of Ncam1 and Cdh4 in the mutant BGCs at P7 is suggested to be the reason of maintaining their active migration and further movement from pcl. Among all studied CAMs only the expression pattern of Ncam1 and Cdh4 is more in line with my hypothesis. Therefore, it is concluded that Ncam1 and Cdh4 are potentially involved in BGCs-PCs attachment, regulating the coupled migration and ultimately forming the PCs monolayer in cerebellar cortex.

REFERENCES

1. Tong KK, Ma TC, Kwan KM. BMP/Smad signaling and embryonic cerebellum development: stem cell specification and heterogeneity of anterior rhombic lip. Dev Growth Differ. 2015;57(2):121-34.
2. du Plessis AJ, Limperopoulos C, Volpe JJ. Chapter 4 - Cerebellar Development. In: Volpe JJ, Inder TE, Darras BT, de Vries LS, du Plessis AJ, Neil JJ, et al., editors. Volpe's Neurology of the Newborn (Sixth Edition): Elsevier; 2018. p. 73-99.
3. Wang SS, Kloth AD, Badura A. The cerebellum, sensitive periods, and autism. Neuron. 2014;83(3):518-32.
4. Muller F, O'Rahilly R. The human brain at stages 18-20, including the choroid plexuses and the amygdaloid and septal nuclei. Anat Embryol (Berl). 1990;182(3):285-306.
5. Alexander CJ, Hammer JA, 3rd. Optimization of cerebellar purkinje neuron cultures and development of a plasmid-based method for purkinje neuron-specific, miRNA-mediated protein knockdown. Methods Cell Biol. 2016;131:177-97.
6. Barmack NH, Yakhnitsa V. Topsy turvy: functions of climbing and mossy fibers in the vestibulo-cerebellum. Neuroscientist. 2011;17(2):221-36.
7. Kern JK. Purkinje cell vulnerability and autism: a possible etiological connection. Brain Dev. 2003;25(6):377-82.
8. Fatemi SH, Halt AR, Realmuto G, Earle J, Kist DA, Thuras P, et al. Purkinje cell size is reduced in cerebellum of patients with autism. Cell Mol Neurobiol. 2002;22(2):171-5.
9. Ritvo ER, Freeman BJ, Scheibel AB, Duong T, Robinson H, Guthrie D, et al. Lower Purkinje cell counts in the cerebella of four autistic subjects: initial findings of the UCLA-NSAC Autopsy Research Report. Am J Psychiatry. 1986;143(7):862-6.
10. Duenas AM, Goold R, Giunti P. Molecular pathogenesis of spinocerebellar ataxias. Brain. 2006;129(Pt 6):1357-70.
11. Manto MU. The wide spectrum of spinocerebellar ataxias (SCAs). Cerebellum. 2005;4(1):2-6.
12. Manto M, Gandini J, Feil K, Strupp M. Cerebellar ataxias: an update. Curr Opin Neurol. 2020;33(1):150-60.
13. Kemp KC, Cook AJ, Redondo J, Kurian KM, Scolding NJ, Wilkins A. Purkinje cell injury, structural plasticity and fusion in patients with Friedreich's ataxia. Acta Neuropathol Commun. 2016;4(1):53.
14. Chen KA, Lanuto D, Zheng T, Steindler DA. Transplantation of embryonic and adult neural stem cells in the granuloprival cerebellum of the weaver mutant mouse. Stem Cells. 2009;27(7):1625-34.
15. Higuera GA, Iaffaldano G, Bedar M, Shpak G, Broersen R, Munshi ST, et al. An expandable embryonic stem cell-derived Purkinje neuron progenitor population that exhibits in vivo maturation in the adult mouse cerebellum. Scientific Reports. 2017;7(1):8863.
16. Dongmei H, Jing L, Mei X, Ling Z, Hongmin Y, Zhidong W, et al. Clinical analysis of the treatment of spinocerebellar ataxia and multiple system atrophy-cerebellar type with umbilical cord mesenchymal stromal cells. Cytotherapy. 2011;13(8):913-7.
17. Chang YK, Chen MH, Chiang YH, Chen YF, Ma WH, Tseng CY, et al. Mesenchymal stem cell transplantation ameliorates motor function deterioration of spinocerebellar ataxia by rescuing cerebellar Purkinje cells. J Biomed Sci. 2011;18:54.
18. Nuryyev RL, Uhlendorf TL, Tierney W, Zatikyan S, Kopyov O, Kopyov A, et al. Transplantation of Human Neural Progenitor Cells Reveals Structural and Functional Improvements in the Spastic Han-Wistar Rat Model of Ataxia. Cell Transplant. 2017;26(11):1811-21.
19. Watson LM, Wong MMK, Vowles J, Cowley SA, Becker EBE. A Simplified Method for Generating Purkinje Cells from Human-Induced Pluripotent Stem Cells. Cerebellum. 2018;17(4):419-27.

20. Volpe JJ, Volpe JJ. Volpe's neurology of the newborn. Sixth edition. ed. Philadelphia, PA: Elsevier; 2018. xv, 1224 pages p.
21. Sotelo C, Rossi F. Purkinje Cell Migration and Differentiation. In: Manto M, Schmahmann JD, Rossi F, Gruol DL, Koibuchi N, editors. Handbook of the Cerebellum and Cerebellar Disorders. Dordrecht: Springer Netherlands; 2013. p. 147-78.
22. Sillitoe RV, Joyner AL. Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. Annu Rev Cell Dev Biol. 2007;23:549-77.
23. Wassef M, Cholley B, Heizmann CW, Sotelo C. Development of the olivocerebellar projection in the rat: II. Matching of the developmental compartmentations of the cerebellum and inferior olive through the projection map. J Comp Neurol. 1992;323(4):537-50.
24. Paradies MA, Eisenman LM. Evidence of early topographic organization in the embryonic olivocerebellar projection: a model system for the study of pattern formation processes in the central nervous system. Dev Dyn. 1993;197(2):125-45.
25. Rahimi-Balaei M, Bergen H, Kong J, Marzban H. Neuronal Migration During Development of the Cerebellum. Front Cell Neurosci. 2018;12:484.
26. Yamada K, Fukaya M, Shibata T, Kurihara H, Tanaka K, Inoue Y, et al. Dynamic transformation of Bergmann glial fibers proceeds in correlation with dendritic outgrowth and synapse formation of cerebellar Purkinje cells. J Comp Neurol. 2000;418(1):106-20.
27. Hanke S, Reichenbach A. Quantitative-morphometric aspects of Bergmann glial (Golgi epithelial) cell development in rats. A Golgi study. Anat Embryol (Berl). 1987;177(2):183-8.
28. Xu H, Yang Y, Tang X, Zhao M, Liang F, Xu P, et al. Bergmann Glia Function in Granule Cell Migration During Cerebellum Development. Molecular Neurobiology. 2013;47(2):833-44.
29. Das GD. Differentiation of Bergmann glia cells in the cerebellum: a golgi study. Brain Res. 1976;110(2):199-213.
30. Yuasa S, Kawamura K, Kuwano R, Ono K. Neuron-glia interrelations during migration of Purkinje cells in the mouse embryonic cerebellum. Int J Dev Neurosci. 1996;14(4):429-38.
31. Shiga T, Ichikawa M, Hirata Y. A Golgi study of Bergmann glial cells in developing rat cerebellum. Anat Embryol (Berl). 1983;167(2):191-201.
32. Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH, et al. Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. Proc Natl Acad Sci U S A. 2005;102(6):2105-10.
33. Bellamy TC. Interactions between Purkinje neurones and Bergmann glia. The Cerebellum. 2006;5(2):116-26.
34. Elias LA, Wang DD, Kriegstein AR. Gap junction adhesion is necessary for radial migration in the neocortex. Nature. 2007;448(7156):901-7.
35. Famulski JK, Trivedi N, Howell D, Yang Y, Tong Y, Gilbertson R, et al. Siah regulation of Pard3A controls neuronal cell adhesion during germinal zone exit. Science. 2010;330(6012):1834-8.
36. Madruga Acerete D, Morande Lavin G, Martinez Orgado J, Capsir Perez F. [The pediatrician and anorexia nervosa]. An Esp Pediatr. 1991;34(1):62-7.
37. Spray DC, Scemes E, Rozental R, Dermietzel R. CHAPTER 15 - Cell–Cell Communication: An Overview Emphasizing Gap Junctions. In: Byrne JH, Roberts JL, editors. From Molecules to Networks. Burlington: Academic Press; 2004. p. 431-58.
38. Kozoriz MG, Naus CC. Chapter 14 - Gap Junction-Mediated Neuroprotection. In: Dere E, editor. Gap Junctions in the Brain. San Diego: Academic Press; 2013. p. 231-46.
39. Shamir ER, Ewald AJ. Chapter Eleven - Adhesion in Mammary Development: Novel Roles for E-Cadherin in Individual and Collective Cell Migration. In: Yap AS, editor. Current Topics in Developmental Biology. 112: Academic Press; 2015. p. 353-82.

40. Shapiro L, Love J, Colman DR. Adhesion molecules in the nervous system: structural insights into function and diversity. Annu Rev Neurosci. 2007;30:451-74.
41. Hou J. Chapter 2 - Paracellular Channel Formation. In: Hou J, editor. The Paracellular Channel: Academic Press; 2019. p. 9-27.
42. Smith RL, Sivaprasad S, Chong V. Retinal Biochemistry, Physiology and Cell Biology. Dev Ophthalmol. 2016;55:18-27.
43. Henry MCW, Lawrence Moss R. chapter 34 - NECROTIZING ENTEROCOLITIS. In: Holcomb GW, Murphy JP, Ostlie DJ, editors. Ashcraft's Pediatric Surgery (Fifth Edition). Philadelphia: W.B. Saunders; 2010. p. 439-55.
44. Koval M. Chapter 1 - Junctional Interplay in Lung Epithelial Barrier Function. In: Sidhaye VK, Koval M, editors. Lung Epithelial Biology in the Pathogenesis of Pulmonary Disease. Boston: Academic Press; 2017. p. 1-20.
45. Nag S. The Blood–Brain Barrier: An Overview☆. Reference Module in Neuroscience and Biobehavioral Psychology: Elsevier; 2017.
46. Wei Q, Huang H. Chapter Five - Insights into the Role of Cell–Cell Junctions in Physiology and Disease. In: Jeon KW, editor. International Review of Cell and Molecular Biology. 306: Academic Press; 2013. p. 187-221.
47. Diaz-Coranguez M, Liu X, Antonetti DA. Tight Junctions in Cell Proliferation. Int J Mol Sci. 2019;20(23).
48. Chapter 31 - Intercellular Junctions. In: Pollard TD, Earnshaw WC, Lippincott-Schwartz J, Johnson GT, editors. Cell Biology (Third Edition): Elsevier; 2017. p. 543-53.
49. Ivanov AI, Naydenov NG. Chapter Two - Dynamics and Regulation of Epithelial Adherens Junctions: Recent Discoveries and Controversies. In: Jeon KW, editor. International Review of Cell and Molecular Biology. 303: Academic Press; 2013. p. 27-99.
50. Hartsock A, Nelson WJ. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta. 2008;1778(3):660-9.
51. Kawauchi T. Cell adhesion and its endocytic regulation in cell migration during neural development and cancer metastasis. Int J Mol Sci. 2012;13(4):4564-90.
52. Juliano RL. Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. Annu Rev Pharmacol Toxicol. 2002;42:283-323.
53. Lavdas AA, Papastefanaki F, Thomaidou D, Matsas R. Cell adhesion molecules in gene and cell therapy approaches for nervous system repair. Curr Gene Ther. 2011;11(2):90-100.
54. Brackenbury R, Rutishauser U, Edelman GM. Distinct calcium-independent and calcium-dependent adhesion systems of chicken embryo cells. Proc Natl Acad Sci U S A. 1981;78(1):387-91.
55. Redies C, Neudert F, Lin J. Cadherins in Cerebellar Development: Translation of Embryonic Patterning into Mature Functional Compartmentalization. The Cerebellum. 2011;10(3):393-408.
56. Hirano S, Nose A, Hatta K, Kawakami A, Takeichi M. Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. J Cell Biol. 1987;105(6 Pt 1):2501-10.
57. Wu Y, Jin X, Harrison O, Shapiro L, Honig BH, Ben-Shaul A. Cooperativity between trans and cis interactions in cadherin-mediated junction formation. Proc Natl Acad Sci U S A. 2010;107(41):17592-7.
58. Sakisaka T, Takai Y. Cell adhesion molecules in the CNS. Journal of cell science. 2005;118(23):5407-10.
59. Angst BD, Marcozzi C, Magee AI. The cadherin superfamily. Journal of cell science. 2001;114(4):625-6.
60. Cailliez F, Lavery R. Cadherin mechanics and complexation: the importance of calcium binding. Biophys J. 2005;89(6):3895-903.

61. Bienz M. beta-Catenin: a pivot between cell adhesion and Wnt signalling. Curr Biol. 2005;15(2):R64-7.
62. Nishimura EK, Yoshida H, Kunisada T, Nishikawa S-I. Regulation of E- and P-Cadherin Expression Correlated with Melanocyte Migration and Diversification. Developmental Biology. 1999;215(2):155-66.
63. Van Aken E, Papeleu P, De Potter P, De Laey J, Mareel M. Cadherin expression in the eye. BULLETIN-SOCIETE BELGE D OPHTALMOLOGIE. 2000:55-60.
64. Basu R, Taylor MR, Williams ME. The classic cadherins in synaptic specificity. Cell Adh Migr. 2015;9(3):193-201.
65. Nakagawa S, Takeichi M. Neural crest emigration from the neural tube depends on regulated cadherin expression. Development. 1998;125(15):2963-71.
66. Ranscht B. Cadherins: molecular codes for axon guidance and synapse formation. International Journal of Developmental Neuroscience. 2000;18(7):643-51.
67. Redies C, Neudert F, Lin J. Cadherins in cerebellar development: translation of embryonic patterning into mature functional compartmentalization. Cerebellum. 2011;10(3):393-408.
68. Redies C, Takeichi M. Expression of N-cadherin mRNA during development of the mouse brain. Developmental dynamics. 1993;197(1):26-39.
69. Huang J, Sakai R, Furuichi T. The docking protein Cas links tyrosine phosphorylation signaling to elongation of cerebellar granule cell axons. Molecular biology of the cell. 2006;17(7):3187-96.
70. Visel A, Thaller C, Eichele G. GenePaint.org: an atlas of gene expression patterns in the mouse embryo. Nucleic Acids Res. 2004;32(Database issue):D552-6.
71. Lele Z, Folchert A, Concha M, Rauch GJ, Geisler R, Rosa F, et al. parachute/n-cadherin is required for morphogenesis and maintained integrity of the zebrafish neural tube. Development. 2002;129(14):3281-94.
72. Dady A, Blavet C, Duband JL. Timing and kinetics of E-to N-cadherin switch during neurulation in the avian embryo. Developmental Dynamics. 2012;241(8):1333-49.
73. Gartner A, Fornasiero EF, Munck S, Vennekens K, Seuntjens E, Huttner WB, et al. N-cadherin specifies first asymmetry in developing neurons. EMBO J. 2012;31(8):1893-903.
74. Benson DL, Tanaka H. N-cadherin redistribution during synaptogenesis in hippocampal neurons. J Neurosci. 1998;18(17):6892-904.
75. Schrick C, Fischer A, Srivastava DP, Tronson NC, Penzes P, Radulovic J. N-cadherin regulates cytoskeletally associated IQGAP1/ERK signaling and memory formation. Neuron. 2007;55(5):786-98.
76. Revollo L, Kading J, Jeong SY, Li J, Salazar V, Mbalaviele G, et al. N-cadherin restrains PTH activation of Lrp6/beta-catenin signaling and osteoanabolic action. J Bone Miner Res. 2015;30(2):274-85.
77. Chalasani K, Brewster RM. N-cadherin-mediated cell adhesion restricts cell proliferation in the dorsal neural tube. Mol Biol Cell. 2011;22(9):1505-15.
78. Zhang J, Woodhead GJ, Swaminathan SK, Noles SR, McQuinn ER, Pisarek AJ, et al. Cortical neural precursors inhibit their own differentiation via N-cadherin maintenance of beta-catenin signaling. Dev Cell. 2010;18(3):472-9.
79. Lelievre EC, Plestant C, Boscher C, Wolff E, Mege RM, Birbes H. N-cadherin mediates neuronal cell survival through Bim down-regulation. PLoS One. 2012;7(3):e33206.
80. Miyamoto Y, Sakane F, Hashimoto K. N-cadherin-based adherens junction regulates the maintenance, proliferation, and differentiation of neural progenitor cells during development. Cell Adh Migr. 2015;9(3):183-92.
81. Kadowaki M, Nakamura S, Machon O, Krauss S, Radice GL, Takeichi M. N-cadherin mediates cortical organization in the mouse brain. Dev Biol. 2007;304(1):22-33.
82. Rieger S, Senghaas N, Walch A, Koster RW. Cadherin-2 controls directional chain migration of cerebellar granule neurons. PLoS Biol. 2009;7(11):e1000240.

83. Koscielny G, Yaikhom G, Iyer V, Meehan TF, Morgan H, Atienza-Herrero J, et al. The International Mouse Phenotyping Consortium Web Portal, a unified point of access for knockout mice and related phenotyping data. Nucleic Acids Res. 2014;42(Database issue):D802-9.
84. Blackshaw S, Harpavat S, Trimarchi J, Cai L, Huang H, Kuo WP, et al. Genomic analysis of mouse retinal development. PLoS Biol. 2004;2(9):E247.
85. Gerhardt H, Rascher G, Schuck J, Weigold U, Redies C, Wolburg H. R- and B-cadherin expression defines subpopulations of glial cells involved in axonal guidance in the optic nerve head of the chicken. Glia. 2000;31(2):131-43.
86. Andrews GL, Mastick GS. R-cadherin is a Pax6-regulated, growth-promoting cue for pioneer axons. J Neurosci. 2003;23(30):9873-80.
87. Gliem M, Weisheit G, Mertz KD, Endl E, Oberdick J, Schilling K. Expression of classical cadherins in the cerebellar anlage: quantitative and functional aspects. Mol Cell Neurosci. 2006;33(4):447-58.
88. Luckner R, Obst-Pernberg K, Hirano S, Suzuki ST, Redies C. Granule cell raphes in the developing mouse cerebellum. Cell Tissue Res. 2001;303(2):159-72.
89. Wang W, Mullikin-Kilpatrick D, Crandall JE, Gronostajski RM, Litwack ED, Kilpatrick DL. Nuclear factor I coordinates multiple phases of cerebellar granule cell development via regulation of cell adhesion molecules. J Neurosci. 2007;27(23):6115-27.
90. Dickinson ME, Flenniken AM, Ji X, Teboul L, Wong MD, White JK, et al. High-throughput discovery of novel developmental phenotypes. Nature. 2016;537(7621):508-14.
91. Soong BW, Paulson HL. Spinocerebellar ataxias: an update. Curr Opin Neurol. 2007;20(4):438-46.
92. Dueñas AM, Goold R, Giunti P. Molecular pathogenesis of spinocerebellar ataxias. Brain. 2006;129(6):1357-70.
93. Manto M-U. The wide spectrum of spinocerebellar ataxias (SCAs). The Cerebellum. 2005;4(1):2.
94. summary NCfBINc. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] October 2015 [Available from: https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=12564.
95. Korematsu K, Nishi T, Okamura A, Goto S, Morioka M, Hamada J, et al. Cadherin-8 protein expression in gray matter structures and nerve fibers of the neonatal and adult mouse brain. Neuroscience. 1998;87(1):303-15.
96. Liu Q, Duff RJ, Liu B, Wilson AL, Babb-Clendenon SG, Francl J, et al. Expression of cadherin10, a type II classic cadherin gene, in the nervous system of the embryonic zebrafish. Gene Expr Patterns. 2006;6(7):703-10.
97. Brasch J, Katsamba PS, Harrison OJ, Ahlsén G, Troyanovsky RB, Indra I, et al. Homophilic and heterophilic interactions of type II cadherins identify specificity groups underlying cell-adhesive behavior. Cell reports. 2018;23(6):1840-52.
98. Arndt K, Redies C. Restricted expression of R-cadherin by brain nuclei and neural circuits of the developing chicken brain. J Comp Neurol. 1996;373(3):373-99.
99. Suzuki S, Sano K, Tanihara H. Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. Cell Regul. 1991;2(4):261-70.
100. Bekirov IH, Nagy V, Svoronos A, Huntley GW, Benson DL. Cadherin-8 and N-cadherin differentially regulate pre- and postsynaptic development of the hippocampal mossy fiber pathway. Hippocampus. 2008;18(4):349-63.
101. Torres VI, Vallejo D, Inestrosa NC. Emerging Synaptic Molecules as Candidates in the Etiology of Neurological Disorders. Neural Plast. 2017;2017:8081758.
102. Soueid J, Kourtian S, Makhoul NJ, Makoukji J, Haddad S, Ghanem SS, et al. RYR2, PTDSS1 and AREG genes are implicated in a Lebanese population-based study of copy number variation in autism. Sci Rep. 2016;6:19088.

103. Huang TN, Hsueh YP. Brain-specific transcriptional regulator T-brain-1 controls brain wiring and neuronal activity in autism spectrum disorders. Front Neurosci. 2015;9:406.
104. Bourgeron T. The genetics and neurobiology of ESSENCE: The third Birgit Olsson lecture. Nord J Psychiatry. 2016;70(1):1-9.
105. Wolfram T, Spatz JP, Burgess RW. Cell adhesion to agrin presented as a nanopatterned substrate is consistent with an interaction with the extracellular matrix and not transmembrane adhesion molecules. BMC Cell Biol. 2008;9:64.
106. Xu C, Cao H, Zhang F, Cheadle C. Comprehensive literature data-mining analysis reveals a broad genetic network functionally associated with autism spectrum disorder. Int J Mol Med. 2018;42(5):2353-62.
107. Duan X, Krishnaswamy A, De la Huerta I, Sanes JR. Type II cadherins guide assembly of a direction-selective retinal circuit. Cell. 2014;158(4):793-807.
108. Rougon G, Hobert O. New insights into the diversity and function of neuronal immunoglobulin superfamily molecules. Annu Rev Neurosci. 2003;26:207-38.
109. Marzban H, Khanzada U, Shabir S, Hawkes R, Langnaese K, Smalla KH, et al. Expression of the immunoglobulin superfamily neuroplastin adhesion molecules in adult and developing mouse cerebellum and their localisation to parasagittal stripes. J Comp Neurol. 2003;462(3):286-301.
110. Science. AIfB. Allen Developing Mouse Brain Atlas: [Available from: https://mouse.brain-map.org/experiment/show?id=80342134.
111. Reyes AA, Small SJ, Akeson R. At least 27 alternatively spliced forms of the neural cell adhesion molecule mRNA are expressed during rat heart development. Mol Cell Biol. 1991;11(3):1654-61.
112. Magdaleno S, Jensen P, Brumwell CL, Seal A, Lehman K, Asbury A, et al. BGEM: an in situ hybridization database of gene expression in the embryonic and adult mouse nervous system. PLoS biology. 2006;4(4).
113. Petridis AK, Nikolopoulos SN, El-Maarouf A. Physical and functional cooperation of neural cell adhesion molecule and β1-integrin in neurite outgrowth induction. Journal of Clinical Neuroscience. 2011;18(8):1109-13.
114. Chao CC, Ma YL, Chu KY, Lee EHY. Integrin αv and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. Neurobiology of Aging. 2003;24(1):105-16.
115. Halaby DM, Mornon JP. The immunoglobulin superfamily: an insight on its tissular, species, and functional diversity. J Mol Evol. 1998;46(4):389-400.
116. Togashi H, Sakisaka T, Takai Y. Cell adhesion molecules in the central nervous system. Cell Adh Migr. 2009;3(1):29-35.
117. Schachner M, Leshchyns'ka I, Sytnyk V. Neural Cell Adhesion Molecules and Synapse Regulation. In: Squire LR, editor. Encyclopedia of Neuroscience. Oxford: Academic Press; 2009. p. 91-6.
118. Xiao M-F, Xu J-C, Tereshchenko Y, Novak D, Schachner M, Kleene R. Neural cell adhesion molecule modulates dopaminergic signaling and behavior by regulating dopamine D2 receptor internalization. Journal of Neuroscience. 2009;29(47):14752-63.
119. Buttner B, Reutter W, Horstkorte R. Cytoplasmic domain of NCAM 180 reduces NCAM-mediated neurite outgrowth. J Neurosci Res. 2004;75(6):854-60.
120. George KS, Wu S. Lipid raft: A floating island of death or survival. Toxicol Appl Pharmacol. 2012;259(3):311-9.
121. Walmod PS, Kolkova K, Berezin V, Bock E. Zippers make signals: NCAM-mediated molecular interactions and signal transduction. Neurochem Res. 2004;29(11):2015-35.
122. Niethammer P, Delling M, Sytnyk V, Dityatev A, Fukami K, Schachner M. Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis. J Cell Biol. 2002;157(3):521-32.

123. Petridis AK, El-Maarouf A, Rutishauser U. Polysialic acid regulates cell contact-dependent neuronal differentiation of progenitor cells from the subventricular zone. Dev Dyn. 2004;230(4):675-84.
124. Angata K, Huckaby V, Ranscht B, Terskikh A, Marth JD, Fukuda M. Polysialic acid-directed migration and differentiation of neural precursors are essential for mouse brain development. Mol Cell Biol. 2007;27(19):6659-68.
125. Rønn LCB, Hartz BP, Bock E. The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system. Experimental Gerontology. 1998;33(7):853-64.
126. Zhang Y, Zhang X, Yeh J, Richardson P, Bo X. Engineered expression of polysialic acid enhances Purkinje cell axonal regeneration in L1/GAP-43 double transgenic mice. Eur J Neurosci. 2007;25(2):351-61.
127. Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, et al. Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature. 1994;367(6462):455-9.
128. Koirala S, Corfas G. Identification of novel glial genes by single-cell transcriptional profiling of Bergmann glial cells from mouse cerebellum. PLoS One. 2010;5(2):e9198.
129. Raponi E, Agenes F, Delphin C, Assard N, Baudier J, Legraverend C, et al. S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage. Glia. 2007;55(2):165-77.
130. Yuasa S, Kawamura K, Ono K, Yamakuni T, Takahashi Y. Development and migration of Purkinje cells in the mouse cerebellar primordium. Anat Embryol (Berl). 1991;184(3):195-212.
131. Yuasa S. Bergmann glial development in the mouse cerebellum as revealed by tenascin expression. Anat Embryol (Berl). 1996;194(3):223-34.
132. Miyata T, Ono Y, Okamoto M, Masaoka M, Sakakibara A, Kawaguchi A, et al. Migration, early axonogenesis, and Reelin-dependent layer-forming behavior of early/posterior-born Purkinje cells in the developing mouse lateral cerebellum. Neural Dev. 2010;5:23.
133. Fisher M. Neuronal influence on glial enzyme expression: evidence from mutant mouse cerebella. Proc Natl Acad Sci U S A. 1984;81(14):4414-8.
134. Bellamy TC. Interactions between Purkinje neurones and Bergmann glia. Cerebellum. 2006;5(2):116-26.
135. Li K, Leung AW, Guo Q, Yang W, Li JY. Shp2-dependent ERK signaling is essential for induction of Bergmann glia and foliation of the cerebellum. J Neurosci. 2014;34(3):922-31.
136. Chao CC, Ma YL, Chu KY, Lee EH. Integrin alphav and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. Neurobiol Aging. 2003;24(1):105-16.
137. Cunningham BA, Hemperly JJ, Murray BA, Prediger EA, Brackenbury R, Edelman GM. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science. 1987;236(4803):799-806.
138. Bi JJ, Li J, Cheng BF, Yang HJ, Ding QQ, Wang RF, et al. NCAM affects directional lamellipodia formation of BMSCs via beta1 integrin signal-mediated cofilin activity. Mol Cell Biochem. 2017;435(1-2):175-83.
139. Diestel S, Hinkle CL, Schmitz B, Maness PF. NCAM140 stimulates integrin-dependent cell migration by ectodomain shedding. J Neurochem. 2005;95(6):1777-84.
140. Edvardsen K, Pedersen PH, Bjerkvig R, Hermann GG, Zeuthen J, Laerum OD, et al. Transfection of glioma cells with the neural-cell adhesion molecule NCAM: effect on glioma-cell invasion and growth in vivo. Int J Cancer. 1994;58(1):116-22.
141. Gratsa A, Rooprai HK, Rogers JP, Martin KK, Pilkington GJ. Correlation of expression of NCAM and GD3 ganglioside to motile behaviour in neoplastic glia. Anticancer Res. 1997;17(6B):4111-7.
142. Schmid RS, Maness PF. L1 and NCAM adhesion molecules as signaling coreceptors in neuronal migration and process outgrowth. Curr Opin Neurobiol. 2008;18(3):245-50.

143. Guan F, Wang X, He F. Promotion of cell migration by neural cell adhesion molecule (NCAM) is enhanced by PSA in a polysialyltransferase-specific manner. PLoS One. 2015;10(4):e0124237.
144. Sur S, Guler MO, Webber MJ, Pashuck ET, Ito M, Stupp SI, et al. Synergistic regulation of cerebellar Purkinje neuron development by laminin epitopes and collagen on an artificial hybrid matrix construct. Biomater Sci. 2014;2(6):903-14.
145. Prakasam AK, Maruthamuthu V, Leckband DE. Similarities between heterophilic and homophilic cadherin adhesion. Proc Natl Acad Sci U S A. 2006;103(42):15434-9.
146. Shan WS, Tanaka H, Phillips GR, Arndt K, Yoshida M, Colman DR, et al. Functional cis-heterodimers of N- and R-cadherins. J Cell Biol. 2000;148(3):579-90.
147. Bouslama-Oueghlani L, Wehrle R, Doulazmi M, Chen XR, Jaudon F, Lemaigre-Dubreuil Y, et al. Purkinje cell maturation participates in the control of oligodendrocyte differentiation: role of sonic hedgehog and vitronectin. PLoS One. 2012;7(11):e49015.
148. Heng C, Lefebvre O, Klein A, Edwards MM, Simon-Assmann P, Orend G, et al. Functional role of laminin alpha1 chain during cerebellum development. Cell Adh Migr. 2011;5(6):480-9.
149. Nishimura EK, Yoshida H, Kunisada T, Nishikawa SI. Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. Dev Biol. 1999;215(2):155-66.
150. Van Aken E, Papeleu P, De Potter P, De Laey JJ, Mareel M. Cadherin expression in the eye. Bull Soc Belge Ophtalmol. 2000(278):55-9.
151. Franco SJ, Muller U. Extracellular matrix functions during neuronal migration and lamination in the mammalian central nervous system. Dev Neurobiol. 2011;71(11):889-900.
152. Lefort CT, Wojciechowski K, Hocking DC. N-cadherin cell-cell adhesion complexes are regulated by fibronectin matrix assembly. J Biol Chem. 2011;286(4):3149-60.
153. Govern CC, Chakraborty AK. Signaling cascades modulate the speed of signal propagation through space. PLoS One. 2009;4(2):e4639.
154. Locasale JW. Signal duration and the time scale dependence of signal integration in biochemical pathways. BMC Syst Biol. 2008;2:108.
155. Camand E, Peglion F, Osmani N, Sanson M, Etienne-Manneville S. N-cadherin expression level modulates integrin-mediated polarity and strongly impacts on the speed and directionality of glial cell migration. J Cell Sci. 2012;125(Pt 4):844-57.