The regulatory roles of TGF-β_1 in formation of the cerebellar primordium during early developmental stages

By

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Abstract

Coordinated production of the multiple neural cell types within the cerebellar primordium is critical during cerebellum development. All the GABAergic and Glutamatergic neurons are derived from the cerebellar ventricular zone and rhombic lip, respectively. Purkinje cells (PCs) and Cerebellar nuclei (CN) neurons are among the earliest neurons born approximately between embryonic days (E) 9 to 13. Before embryonic day 14.5, postmitotic and differentiated PCs and CN neurons migrate towards the PC plate (PCP) and nuclear transitory zone (NTZ) of the cerebellar primordium. The cellular and molecular mechanisms underlying the early cerebellar neurogenesis, migration/differentiation, and connectivity establishment are unclear. Macroautophagy (hereafter autophagy) plays an essential role in regulating cellular phenotype, including epithelial to mesenchymal transition and endothelial to mesenchymal transition. Transforming growth factor-beta 1 (TGF-β_1) is also involved in regulating cellular phenotype via several mechanisms, including autophagy. It is a key player in pre-and postnatal development. Therefore, we hypothesis that TGF-β_1 may control early cerebellar development by modulating the levels of cell adhesion molecules (CAMs) and autophagy pathway in the mouse cerebellar primordium. To better understand the role of TGF-β_1, we used mouse embryonic cerebellar tissues derived from embryonic days 9 to 13, performed RT-qPCR, western blotting, and analyzed in situ hybridization (ISH) data; from "Allen Developing Mouse Brain Atlas." In this study, I showed the activation of the Canonical TGF-β signaling pathway at the time window that coincides with the formation of the PCP and NTZ. In addition, my data demonstrate that activated TGF-β signaling pathway sequentially and temporally could upregulate the expression of N-cadherin and β-catenin with maximum expression at E11/E12, with subsequent upregulation of the Cdh8 and NCAM expression at E12 and E13. My data also showed activated TGF-β signaling occurs concurrently with inhibition of autophagic-flux at E11/E12. However, basal autophagy occurs during earlier developmental stages from E9 to E10. This study identified a crucial role of the TGF-β signaling pathway and its regulatory effects on Cadherins expression and autophagic flux during cerebellar development, which all together potentially contribute to the proliferation, migration/differentiation, and positioning of the cerebellar nuclei neurons and Purkinje cells within their domains.

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DEDICATION

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACC: animal care committee

Allen DMBA: Allen developing mouse brain atlas

ASD: autism spectrum disorders

ATGs: autophagy-related proteins

Atoh1: atonalhomolog 1

BBB: blood brain barrier

BDNF: brain-derived neurotrophic factor

BG: bergmann glia

BMP: bone morphogenic protein

BSA: bovine serum albumin

Calbindin: calcium binding protein

CAMs: cell adhesion molecules

Cdh: cadherin

CCAC: Canadian council on animal care

cDNA: complementary DNA

CN: cerebellar nuclei

CNS: central nervous system

Co-Smad: common partner Smad

ECM: extracellular matrix

EGL: external granular layer

EMT: epithelial mesenchymal transition

ER: Endoplasmic Reticulum

FGF8: fibroblast growth factor 8

4^{th} ventricle: fourth ventricle

GABAergic: gamma-butyric acid

GCPs: granule cells precursor

GDF: growth and differentiation factor

GL: granule cell layer

Gbx2: gastrulation brain homeobox

IL: interleukin

ISH: in situ hybridization

I-Smad: inhibitory Smad

ISO: isthmic organizer

kDa: Kilo Dalton

LAP: latency associated peptide

LC3: light chain 3

LLC: large latent complex

Lmx1α: Lim homeobox transcription factor 1 alpha

LTBP: latent TGF-β binding protein

MAPK: mitogen activated protein kinases

MCP: middle cerebellar peduncle

MET: Mesenchymal to Epithelial Transition

MHB: mid-hindbrain boundary

ML: molecular layer

NCAM: neural cell adhesion molecule

NTZ: nuclear transitory zone

Otx2: orthodenticle homologue 2

P: postnatal

PCD: programmed cell death

Pcl: purkinje cell layer

PCP: purkinje cell plate

PCR: polymerase chain reaction

PCs: purkinje cells

Ptf1α: pancreas transcription factor 1 subunit alpha

RT-qPCR: reverse transcription- quantitative PCR

R-Smad: receptor regulated Smad

SARA: Smad anchor for receptor activation

SBE: Smad binding elements

Shh: sonic hedgehog

SP: signal peptide

SLC: small latent TGF-β complex

TGFβRI & II: type 1 & 2 TGF-β receptors

TGF-β: transforming growth factor beta

URL: upper rhombic lip

VZ: ventricular zone

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: INTRODUCTION

1.1 CEREBELLUM

1.1.1 Anatomy & Organization

The cerebellum, also known as the "Little Brain," is located as a distinct subdivision inferior to the occipital lobe of the brain and posterior to the brain stem and fourth ventricle (Glickstein et al., 2009; Jimsheleishvili & Dididze, 2019). The Cerebellum, Pons, and Medulla oblongata form the hindbrain of the adult brain that initially developed from the rhombencephalon during central nervous system (CNS) embryological development (Hibi and Shimizu 2012). The cerebellum is connected to the brain stem (midbrain, pons, and medulla) through the big mass of myelinated axons called cerebellar peduncles including superior, middle, and inferior peduncles (Jimsheleishvili and Dididze 2019). These peduncles contain ascending and descending tracts that act as bridges between the cerebellum and different parts of the brain (Jimsheleishvili & Dididze, 2019; Nagahama et al., 2021).

The anatomical structure of the mammalian cerebellum is characterized by two laterally large hemispheres which are united in the structure at the midline called the vermis (Jimsheleishvili & Dididze, 2019; Marzban et al., 2015). The cerebellum contains 80% of the brain's neurons that are aggregated in a cerebellar cortex with less than 1mm thick (Jimsheleishvili and Dididze 2019). Due to the convolution, the surface of both hemispheres and the vermis is divided into three distinct lobes including anterior, posterior, and flocculonodular by two main transverse fissures: primary and posterolateral fissures. In addition, further subdivided into ten lobules (I-X) and each lobule into numerous folia (Basson & Wingate, 2013; Jimsheleishvili & Dididze, 2019; Van Essen et al., 2018).

Coronal sectioning through the cerebellar hemispheres reveals major internal subdivisions; folded gray matter that surrounds the highly branched inner white matter at the core of the cerebellum (Jimsheleishvili & Dididze, 2019; Van Essen et al., 2018). Cerebellar gray matter is found in both inner and outer zones called cerebellar nuclei and cerebellar cortex, respectively (Jimsheleishvili & Dididze, 2019; Marzban et al., 2015). Three pairs of cerebellar nuclei including dentate, fastigial, and interposed consisting of emboliform and, globose are situated at the core of

the cerebellum and deep within the white matter (Cacciola et al. 2019). Cerebellar nuclei structures have specific connections with the cerebellar cortical zones (both hemispheres and vermis) that finally lead to specific motor responses such as coordination and balance in upper motor neuronal systems (Uusisaari and de Schutter 2011). The cerebellar cortex, the outer gray zone, is the highly convoluted region that is formed by three distinct cellular layers located just around the white matter including Molecular layer (ML), Purkinje layer (PCL), and Granular layer (GL) (Jimsheleishvili & Dididze, 2019; Marzban et al., 2015). White matter which contains myelinated axons, provides afferent and efferent innervations for all the cell types located in both cerebellar nuclei and cerebellar cortex (Van Essen, Donahue, and Glasser 2018).

1.1.2 Cytoarchitecture

Cerebellar nuclei neurons are situated within the white matter and adjacent to the 4th ventricle's roof plate (Marzban et al. 2015). Cells within the three-layered cerebellar cortex are distributed across three different layers as follows. The ML is the external and superficial cellular layer of the cerebellar cortex which contains dense dendritic arborization of the Purkinje cells (PCs) that their cell bodies are originally situated in the middle PCL. Another hallmark of this layer is the granule cells' axon extending from the GL. Together, these axons form the parallel fibers within the dense arbors of hundred Purkinje cells (Consalez et al. 2021a). Very few cell types, such as Stellate and Basket cells, are localized in the molecular layer. The second and middle layer, just beneath the molecular layer, is the Purkinje cell layer composed of the Candelabrum cells and large cell bodies of the PCs that extend their arbors to the molecular layer. The last and internal layer superficial to the white matter is the granular cell layer, known for the highly packed and dense organized cell bodies of the granule cells. This layer also contains other cell types including Golgi cells, Lugaro cells, and unipolar brush cells (Jimsheleishvili & Dididze, 2019; Marzban et al., 2015). In addition, axons of the PCs are passed through the granular layer to reach the cerebellar nuclei neurons (Consalez et al., 2021a; Jimsheleishvili & Dididze, 2019). Cerebellar nuclei and cortical neurons contain few cell types receiving excitatory and inhibitory impulses that all together form the cerebellar circuit (Cacciola et al. 2019).

1.1.3 Cerebellar Circuit & Function

Cerebellum is all about execution and fine coordination of motor movements, posture, and balance (Hibi & Shimizu, 2012; Jimsheleishvili & Dididze, 2019; Marzban et al., 2015). In addition, cerebellum plays an important role in higher cognitive function such as attention, language, sleep, and emotional behaviour (Hibi et al., 2017; Hibi & Shimizu, 2012; Marzban et al., 2015). These vital functions highly dependent on formation of the precise and accurate cerebellar circuit (Hibi and Shimizu 2012). Briefly, in our body, all the major voluntary and motor pathways are initiated in the pre-central gyrus of the cerebral cortex, which is the primary motor cortex. Motor neurons in the primary motor cortex form the corticospinal tracts that extend down through the brain stem, project to the pons, and transfer to the cerebellum by cerebellar peduncles. Neural information is processed in the cerebellar cortex, and consequently, the output is transferred to the cerebellar nuclei. Projections from the cerebellar nuclei innervate the thalamus, which relays motor information. All the motor inputs from the cerebellar nuclei to the thalamus are relayed back to the upper motor neurons in the cerebral primary motor cortex. Descending tracts from the primary motor cortex enter the ventral horn of the spinal cord, synapse with lower motor neurons in the ventral horn, and activate them. Finally, motor signals extend out of the spinal cord through the ventral root and innervate to the skeletal muscles, which lead to the contraction response. In this loop, the cerebellum does not play a role as a movement initiator; however, the cerebellum is responsible for coordinating movement and controlling balance during activated muscular response. So, cerebellar damage is not associated with paralysis, and people can still move without the cerebellum; however, they experience uncoordinated movements and loss of posture and balance (Jimsheleishvili & Dididze, 2019; Roland & Zilles, 1998; Sheets & Shepherd, 2011).

1.1.4 Cerebellar afferents

Three kinds of inputs arrive in both cerebellar nuclei and cerebellar cortex through cerebellar peduncles: including climbing fibers originating from inferior olivary nuclei neurons, which receive inputs from the cerebral cortex, spinal cord and brain stem; mossy fibers from multiple sources in the central nervous system such as the vestibular nuclei; and finally locus coeruleus noradrenergic and varicose arborizations of the monoaminergic fibers (Hibi & Shimizu,

2012; Marzban et al., 2015). These afferents carry information related to the voluntary movements and balance from the cerebral cortex, muscles, tendons, joints, and vestibular nuclei in the ipsilateral manner which means from the same side of the body (Jimsheleishvili and Dididze 2019).

Climbing and mossy fibers are the primary and major excitatory inputs to the cerebellar cortex originating from neurons located in surrounding nuclei which are referred to as pre-cerebellar nuclei (Hibi et al., 2017; Hibi & Shimizu, 2012; Jimsheleishvili & Dididze, 2019). These neurons also send collateral branches to the cerebellar nuclei neurons (Hibi and Shimizu 2012). The climbing fibers are initially axons of the inferior olivary nuclei neurons that use glutamate as the excitatory neurotransmitters (Hibi & Shimizu, 2012; Jimsheleishvili & Dididze, 2019; Marzban et al., 2015). These glutamatergic projections travel in the cortex to the molecular layer, wrap around the PCs, and make excitatory synaptic connections with the soma and dendritic trees of the PCs (Hibi et al., 2017; Jimsheleishvili & Dididze, 2019; Marzban et al., 2015). The mossy fibers are derived from multiple nuclei within the brain stem and spinal cord (Hibi and Shimizu 2012). Like climbing fibers, mossy fibers are excitatory projections that synapse with dendrites of the granule cells in the granular cell layer (Hibi et al., 2017; Jimsheleishvili & Dididze, 2019). Consequently, to convey all the external and internal information, axons of the granule cells use glutamate and innervate to the molecular layer within the dendritic arborization of the PCs and form arrays of parallel fibers (Hibi et al., 2017; Hibi & Shimizu, 2012; Marzban et al., 2015). Each parallel fiber makes synapses with abundant PCs via multiple branching (Jimsheleishvili and Dididze 2019). Postsynaptic connections on PCs lead to integrating all the information received from both excitatory climbing and mossy fibers (Hibi and Shimizu 2012). Climbing fiber/parallel fiber conjunction results in activation of Ca2+ channels throughout PCs dendrites and consequently large Ca2+ influx into the PCs, which lead to depolarization and electrical inhibition of the PCs signals referring to as long term depression (LTD) (Hansel & Bear, 2007; Hibi & Shimizu, 2012; Jimsheleishvili & Dididze, 2019). In turn, PCs axon projections as the sole output of the cerebellar cortex provide inhibitory impulses to the cerebellar nuclei neurons using gamma-aminobutyric acid (GABA) as the neurotransmitter. In addition, axons of some PCs send a direct branch to the balance center located in the hindbrain called vestibular nuclei (Hibi et al. 2017). Finally, in an ipsilateral manner, projections from the cerebellar nuclei are relayed through the thalamus and then sent back to upper motor neurons located in the cerebral primary motor cortex through the cerebellar peduncles (Jimsheleishvili & Dididze, 2019; Marzban et al., 2015).

The last set of afferent fibers to the cerebellar cortex originates from neurons located in the locus coeruleus that send their noradrenergic axons to all three layers of the cerebellar cortex (Marzban et al. 2015). All the described inputs and outputs to and from the cerebellum make a precise circuit. This circuit process receiving peripheral and major information, which altogether provide coordinated fine movements and balance (Glickstein et al., 2009; Hansel & Bear, 2007; Hibi & Shimizu, 2012; Jimsheleishvili & Dididze, 2019; Marzban et al., 2015).

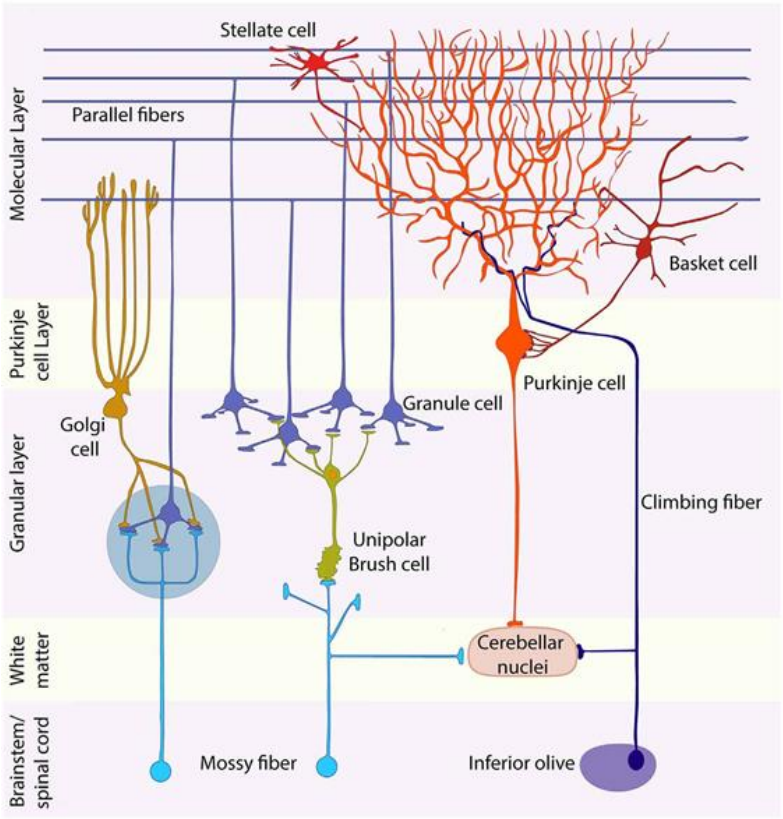


Figure 1. Schematic illustration of the cerebellar circuit (Consalez et al. 2021a). All the cerebellar neurons are distributed across three distinct cellular layers including: Molecular layer, Purkinje cell layer, and Granular layer. Two major fibers, Mossy and Climbing fibers, project into the cerebellum that provide excitatory impulses for Granule cells and dendritic arborization of the Purkinje cells, respectively. Both fibers send direct branches to cerebellar nuclei located in the white matter. Granule cells use glutamate and extend their fibers into the molecular layer, then bifurcate and form the parallel fibers which make abundant synaptic connections with the dendritic tree of the PCs. Finally, PCs as the sole output of the cerebellum, provides inhibitory impulses for cerebellar nuclei.

1.1.5 Development of the Cerebellum

In humans and rodents, the formation of the neural tube starts during the embryonic period follows by differentiation and maturation in the fetal and postnatal period (Dessaud, McMahon, and Briscoe 2008). Table 1 shows the comparative timing of development between human, rat, and mouse. In a human embryo, 18 days after fertilization, notochord develops along the midline of the embryonic plate as a flat structure and serves as the basis for developing the axial skeleton (le Dréau and Martí 2012). As the notochord forms, it produces concentration gradients of signaling molecules such as Shh that induce differentiation of the overlying epithelial layer into the neuroepithelium/neural plate and adjacent cells into the neural crest cells which will become different cell types of the central and peripheral nervous system (Dessaud et al., 2008; Le Dréau & Martí, 2012; Nikolopoulou et al., 2017). During the process called invagination, neuroepithelium begins to fold inward and role over that form the neural groove, and finally leads to the formation of the neural tube in the middle of the embryo, which is the primordium of the central nervous system (le Dréau and Martí 2012). Approximately four weeks after fertilization (24-28 days), the neural tube gradually fuses rostrally and caudally. It starts to close at three closure sites including anterior neuropore near the forehead, posterior/lumbar neuropore down at the tail, and rhombencephalic neuropore in the occipital region at the back of the head. These processes, also known as neurulation, are completed by the end of the fourth gestation week (Nikolopoulou et al. 2017). At the end of the embryonic period (8 weeks), rostral-caudal expression of homeobox genes induce longitudinal segmentation of the neural tube and formation of the bulges and rudimentary components of the developing CNS including Prosencephalon, refers to the future forebrain, which is further subdivided into Telencephalon and Diencephalon (Dessaud, McMahon, and Briscoe 2008). Later, these structures form the cerebral hemispheres and thalamus/hypothalamus, respectively. Mesencephalon or Midbrain, Rhombencephalon/Hindbrain including Metencephalon (pons and cerebellum) and Myelencephalon (medulla), and spinal cord are the other parts of the CNS that form during human embryonic period (le Dréau and Martí 2012; Nikolopoulou et al. 2017).

Table 1. Comparative development timing

Species	Embryonic period	Gestation (Brain size)	Mature (Brain size)
Human	8 weeks	37-41 weeks (~ 30%)	10 years
Rat	17.5 days	21-23 days (~ 15%)	60 days
Mouse	16 days	19-21 days (~ 25%)	45 days

1.1.6 Cerebellar Primordium

Neural tube patterning occurs in three axes including mediolateral (formation of the neural tube/ neurulation), dorso-ventral (dorsalization and ventralization of the spinal cord, rhombencephalon, and mesencephalon), and rostrocaudal/anteroposterior (rhombencephalon and mesencephalon) axis. By 12 weeks gestation (early fetal period), development of the cerebellar bulges starts in the rhombencephalon region, dorsal part of the rostral most hindbrain (Hibi et al., 2017; Hibi & Shimizu, 2012; Keefe & Nowakowski, 2020). During anteroposterior patterning, the rhombencephalon is divided into eight rhombomeres (r1-r8). In addition, anteroposterior patterning of the neural tube causes formation of the isthmus, narrow constriction of tissue at the junction of the midbrain and hindbrain, also known as mid-hindbrain boundary (MHB) (Hibi et al., 2017; Hibi & Shimizu, 2012). This boundary contains an isthmic organizer (ISO), which is the secondary organizer and signaling center with long range effects on the fate of rostrally and caudally surrounding tissues including mesencephalon and metencephalon (Hibi & Shimizu, 2012; Leto et al., 2016). Cerebellar primordium is derived from the dorsal part of the rhombomere 1 (r1), the rostral-most segment of the hindbrain, under organizing activity of the ISO (Di Giovannantonio et al., 2014; Keefe & Nowakowski, 2020). Development, neurogenesis, and fate

decisions of cerebellar primordium cells are dependent on the expression of ISO signaling molecules (Hibi & Shimizu, 2012; Leto et al., 2016; Marzban et al., 2015).

1.1.7 Transcription Factors

After neural tube closure at the end of neurulation, cells in the anterior and posterior neural tube express two homeodomain transcription factors including orthodenticle homologue 2 (Otx2) and gastrulation brain homeobox (Gbx2) (Hibi & Shimizu, 2012; Leto et al., 2016). Combined expression pattern of these transcription factors in two anterior and posterior complementary domains lead to interface of the Otx2/Gbx2 positive neuroepithelial cells and consequent formation and positioning of the MHB which contains a ring of organizing cells called isthmic organizer (ISO) (Basson & Wingate, 2013; Di Giovannantonio et al., 2014; Hibi et al., 2017; Hibi & Shimizu, 2012; Leto et al., 2016). Target progenitor domains by interpreting signaling molecules, producing by ISO, establish mesencephalon and rhombencephalon, future midbrain and cerebellum, at the rostral and caudal parts of the defined boundary (Di Giovannantonio et al., 2014; Hibi et al., 2017; Leto et al., 2016).

Among these homeobox genes, Otx2 expression occurs first and throughout the entire trilaminar germ layers at the forebrain and midbrain (Matsuo et al. 1995). Otx2 gene, also known as a head organizer during embryonic development, is expressed at embryonic day 7.5 (E7.5) in the mouse embryo rostral head that plays an important role in patterning the rostral territories including forebrain and craniofacial structures (Di Giovannantonio et al., 2014; Matsuo et al., 1995). Matsuo et al., by generating *Otx2* mutant mice with otocephalic phenotype, examined the role of *Otx2* gene expression in the development of the rostral and caudal portions of the head. Results showed that defects in the *Otx2* gene cause anomalies in more rostral regions of the head with no defects in caudal portion including hindbrain. The absence of rostral head structures in *Otx2* mutant mouse indicates the importance of the *Otx2* gene expression during formation and proper development of the rostral head (di Giovannantonio et al. 2014). In this context, further studies on the neuroectoderm cells identified that rostrally expression of Otx2 specifies the mesencephalic territory and fate of dorsal mesencephalic progenitors. In contrast, specific amounts of Gbx2 in caudal regions is needed for regulating the patterning of the rhombencephalon domains

(Di Giovannantonio et al., 2014; Hibi et al., 2017; Matsuo et al., 1995). A wealth of data has indicated that lacking expression of Otx2 in the caudally rhombencephalon regions, where required threshold amount of Gbx2 is present, cause the formation of the mouse cerebellar primordium at embryonic day 7-8 (Chizhikov & Millen, 2020; Marzban et al., 2015).

At the junction of the Otx2 and Gbx2 positive cells, where organizing centers are localized, other transcription factors are expressed in a spatiotemporal pattern and at specific developmental stages of the mesencephalon and cerebellar territories (Basson & Wingate, 2013; Di Giovannantonio et al., 2014; Leto et al., 2016). Transcription factors including fibroblast growth factor 8 (Fgf8), Wnt1, Lmx1b, Pax2/5, and Engrailed1/2 (Eng1/2) are expressed in the isthmic organizer region that determine and specify the fate of target progenitor cells in the rostrally and caudally surrounding areas (Chizhikov & Millen, 2020; Di Giovannantonio et al., 2014; Hibi et al., 2017). In addition, these set of transcription factors together form a network that contributes to the formation, activation, and maintenance of the ISO (Chizhikov & Millen, 2020; Hibi et al., 2017; J Xu et al., 2000).

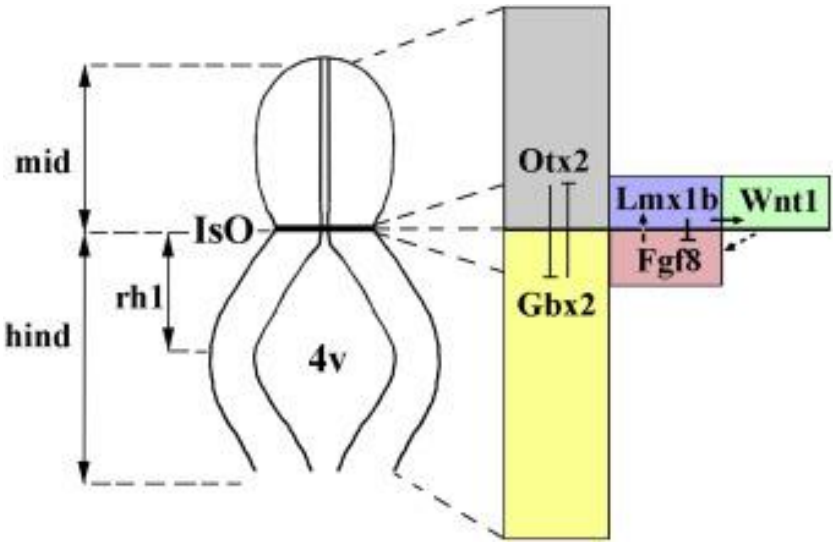


Figure 2. Transcription factors in cerebellar Neurogenesis (Chizhikov and Millen 2020). Expressed transcription factors in the ISO including Fgf8, Wnt1, and Lmx1b specify the fate of target progenitor cells locating in the rostral and caudal regions of the MHB. Fgf8 induce and maintain the expression of Wnt1 and Lmx1b, while Lmx1b and Wnt1 regulate initiation and maintenance of the Fgf8 expression.

After formation of the cerebellar primordium (E7-8), due to the repression of Otx2 and expression of Gbx2 in caudal regions, at E8.5 key organizing molecule, Fgf8 is secreted by the ISO with higher amounts of expression in the rostral rhombencephalon, enriched with Gbx2 expressing cells (Basson & Wingate, 2013; Di Giovannantonio et al., 2014; Leto et al., 2016). The decreasing gradient of Fgf8 protein by repressing *Otx2* gene plays an important role in enforcing MHB, cerebellar neurons fate decision and cerebellar morphogenesis (Di Giovannantonio et al., 2014; Hibi et al., 2017; Leto et al., 2016; J Xu et al., 2000). It has been reported that by placing Fgf8 beads in the more anterior mesencephalic regions of the neural tube, an ectopic MHB formed, resulting in the formation of new midbrain and hindbrain locating in more anterior regions of the neural tube (J Xu, Liu, and Ornitz 2000). In addition, further studies on the *Fgf8* gene demonstrated that a partial *Fgf8* gene loss of function caused cell death of all progenitors and lack of midbrain and cerebellar structures formation in mouse models (Chi et al., 2003; Meyers et al., 1998). Together, all these data suggest that Fgf8 acts as a survival factor for all progenitors and plays an essential role in mediating MHB organizing function that could mimic MHB activity when located ectopically (Basson & Wingate, 2013; J Xu et al., 2000).

Development of the MHB along the anterior-posterior axis of the neural tube is highly regulated by genetic programs in which *Fgf8* interacts with a set of organizing genes that form specialized signaling centers called secondary organizers (Basson & Wingate, 2013; J Xu et al., 2000). Before embryonic day 9.5 (E8.5-9.5), *Fgf8* induces and maintains the expression of other signaling molecules necessary to specify the fate of rostrally and caudally located progenitor cells, including Wnt1, Lmx1b, Pax2/5, and Eng1/2 (J Xu, Liu, and Ornitz 2000). Among all the secondary signaling molecules, Wnt1 and Lmx1b are co-expressed in the midbrain, where Otx2 positive cells are located, and regulate cell proliferation by preventing apoptosis in the progenitor cells in both the midbrain and cerebellum (Hibi et al., 2017; Leto et al., 2016). Interestingly, Lmx1b and Wnt1 regulate the initiation and maintenance of the Fgf8 expression, respectively (Basson and Wingate 2013). Hence, any alteration or deregulation of these genes leads to massive disruption in the expression amounts of other factors and consequent malformation/malfunction of the midbrain and hindbrain (di Giovannantonio et al. 2014).

1.1.8 Germinal Zones

The astonishing number of the human neurons (%80), which are located in the cerebellum generating from four neural progenitor domains including two major germinal zones: the cerebellar ventricular zone (VZ) located internally and the upper rhombic lip (URL) at the caudomedial region of the cerebellum (Basson & Wingate, 2013; Hibi et al., 2017; Keefe & Nowakowski, 2020; Vriend et al., 2015), and two outward germinal zones: the external granular layer (EGL) and the rostrally located mesencephalon (Vriend, Ghavami, and Marzban 2015). In mice, cerebellar neurogenesis in germinal zones starts during the embryonic period at about E7 to E13/14, and developmental stages continue postnatally until adulthood (postnatal days) (Hata & Chen, 2016; Vriend et al., 2015).

The cerebellar VZ neural progenitors by expressing Ptf1a (pancreas Transcription factor1 subunit alpha) is the main source of all the inhibitory gamma- butyric acid (GABAergic) neurons in the mammalian cerebellum including Purkinje and Candelabrum cells located in the Purkinje cell layer, Golgi and Lugaro cells in the granular layer, and basket and stellate cells in the molecular layer (Hibi & Shimizu, 2012; Keefe & Nowakowski, 2020; Marzban et al., 2015; Vriend et al., 2015). Under control of NOTCH1 and PTF1a pathways, PCs, which are among the earliest born cerebellar neurons (E10-E13 in mice, 8 weeks post-conception/pcw in human), are generated from the cerebellar VZ located at neuroepithelium/roof of the 4th ventricle (Basson & Wingate, 2013; Hibi et al., 2017; Keefe & Nowakowski, 2020; Marzban et al., 2015; Vriend et al., 2015). As the cerebellar cortex's principal neurons, PCs are the primary source of mitogen that stimulates mitosis in the granule cell precursors located in the EGL (Basson & Wingate, 2013; Marzban et al., 2015). Consequently, genetic deletion of *Ptf1a* leads to complete loss of GABAergic neurons and ectopic formation of the glutamatergic neurons from the mouse cerebellar VZ (Hibi et al. 2017). Other studies demonstrated that genetic disruption of the *Ptf1a* causes severe cerebellar hypoplasia due to the absence of PCs and consequent deficiency of the GCPs proliferation, which all together affects postnatal cerebellar growth (Basson and Wingate 2013) In addition to the GABAergic neurons, Bergmann glia fibers (BG) have emerged from the cerebellar VZ (Basson and Wingate 2013). After the proliferation of the PCs in the mouse cerebellar VZ, at E13-14, PCs transit from proliferative to migratory state (Basson & Wingate, 2013; J Xu et al., 2000). At E18.5 PCs starts to express Sonic hedgehog (Shh) that postnatally affects on GCPs proliferative activity

(Vriend, Ghavami, and Marzban 2015). Using Bergmann glia fibers as the scaffold, PCs migrate radially from the neuroepithelial to Purkinje cell clusters during cerebellar development (Ana P.B. Araujo et al., 2016; Basson & Wingate, 2013). After birth, PCs clusters disperse into the monolayer under the effect of reelin-expressed GCPs (Ana P.B. Araujo et al., 2016; Marzban et al., 2015). As mentioned previously, Fgf8 expression in the mouse cerebellar primordium, which starts at E8.5, terminated at E13-14 which supports the idea that Fgf8 may play a role in regulating the transition of PCs from proliferation to differentiation state (J Xu, Liu, and Ornitz 2000).

The URL is located in the rhombomere 1 of the rhombencephalon domain at the caudomedial region of the cerebellar primordium and develops under control of BMP and LMX1α, which are expressed from the roof plate cells adjacent to the URL (Hibi et al., 2017; Keefe & Nowakowski, 2020; Vriend et al., 2015). Glutamatergic neural progenitors, located in this highly proliferative zone, by expressing Atoh1 (atonal homolog1) create granule cells precursors (GCPs), cerebellar nuclei (CN) projecting neurons, and unipolar brush cells (Basson & Wingate, 2013; Hibi et al., 2017; Keefe & Nowakowski, 2020; Vriend et al., 2015). These atoh1+ progenitor cells are rapidly proliferating cell populations that form the future excitatory glutamatergic neurons (Basson and Wingate 2013). At E12.5-E17, GCPs migrate rostrally and over the surface of the cerebellar primordium to establish the EGL (Vriend, Ghavami, and Marzban 2015). During the early postnatal period at around P4, secreted Shh from the PCs causes extensive proliferation and differentiation of the GCPs in the EGL, which disappears before maturation (Hibi et al., 2017; Marzban et al., 2015; Vriend et al., 2015). Then, they migrate internally through the Bergmann glia fibers and across the PCs layer and reach the granular layer as their destination (Marzban et al. 2015). Therefore, it has been reported that *Atoh1* loss of function cause lack of EGL formation and the consequent absence of mature granule cells (Basson and Wingate 2013). Furthermore, *Atoh1* deletion leads to the ectopic formation of the PCs instead of the glutamatergic neurons from the URL. Evidence shows that *Atoh1* and *Ptf1a* by supressing each other's protein expression in their territories and the absence of the other, contribute to glutamatergic and GABAergic cerebellar cell fate determination in the URL and cerebellar VZ, respectively(Hibi et al. 2017). CN precursors neurons are born between E9.5 to E12.5 from the URL and migrate tangentially towards the nuclear transitory zone (NTZ), forming the future medial, interposed, and lateral cerebellar nuclei neurons located deep within the white matter. Pax6, Tbr2, Tbr1 and Lmx1a are the transcription factors expressed in both URL and NTZ during development of the CN neurons. As shown in

Figure 3, the only neural populations in the cerebellar primordium from E9 to E13 are the CN neurons and PCs (Rahimi-Balaei et al. 2018).

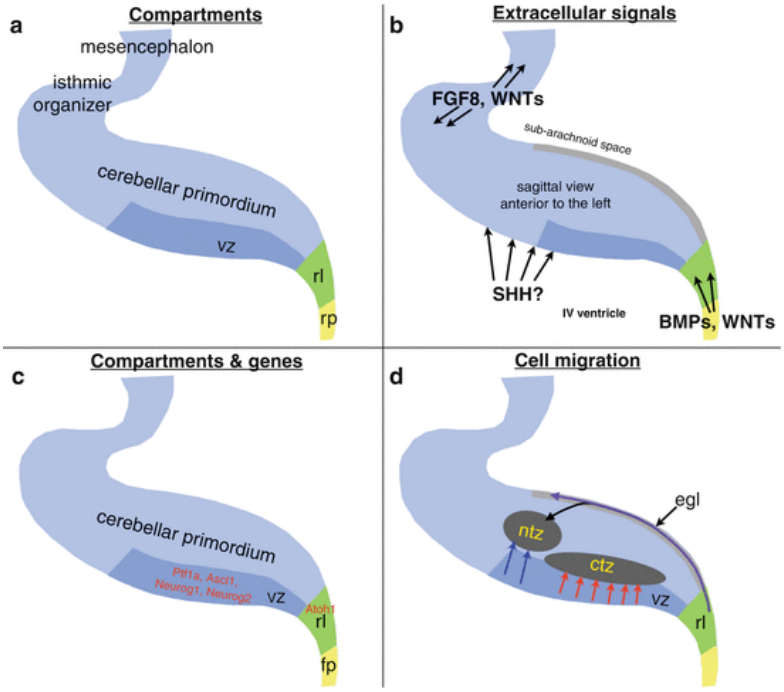


Figure 3. Schematic illustration of the cerebellar primordium with focus on patterning, neurogenesis, and neural migration (Consalez et al. 2013). (a, b) Cerebellar patterning and neurogenesis of the cerebellar primordium which is controlled by function of the isthmic organizer and secretion of the rostrally FGF8/WNT1 and dorsally BMP/WNT, (c) formation of two germinal zones; vz and rl containing gene expression microdomains which adopt GABAergic and glutamatergic neural populations, respectively, (d) radially migrating ventricular zone progenitors, curved arrows indicate granule cell precursors (purple) and cerebellar neuron precursors (black). Abbreviations: ctz cortical transitory zone, egl external granule layer, ntz nuclear transitory zone, rl upper rhombic lip, rp roof plate, vz ventricular zone.

There are key steps during early cerebellar development, including proliferation, neural migration, differentiation, and apoptosis. Disturbances in any of them may lead to neurological problems such as ataxia, intellectual inability, and pediatric tumors. Alteration in cerebellar structure and function leads to emotional, cognition, and social abnormalities in patients with neuropsychiatric disorders such as autism and schizophrenia. The development of the cerebellum is highly controlled by intrinsic genetic programs that are conserved and control the assembly and

accuracy of the cerebellar neural circuits, extracellular signals, and environmental factors (Ana P.B. Araujo et al., 2016; Leto et al., 2016).

So far in this section, we talked about the developmental genes expressing in the cerebellum that contribute and orchestrate the cerebellar primordium and neuronal formation. However, accumulation information show that micro-environmental influences play essential roles in cerebellum and its primordium development. It has been reported that metabolites, cytokines, interleukins, growth factors, hormones, and nutrients by imposing their effects in a spatiotemporal manner contribute to establishing and maintaining the cerebellar circuit and structure. In the following section, we will discuss more about these extracellular factors.

1.2 CYTOKINES: CHEMOKINES & INTERLEUKINS

Cytokine is a general name referred to small, secreted proteins that affect the communications and interactions of the cells in autocrine, paracrine, and endocrine manner (Erta et al., 2012; Zhang & An, 2007). Based on the cell type and the function, cytokines are named differently. For example, cytokines secreted by one leukocyte and effect on other leukocytes are known as interleukins, and cytokines with chemotactic activities are called chemokines. Cytokines produce cascades and stimulate their target cells, cause the secretion of other cytokines (Zhang & An, 2007). Many cell populations produce cytokines; however, helper T cells (Th) and macrophages are the most abundant producers of these secreted proteins (Erta, Quintana, and Hidalgo 2012). There is significant evidence showing that specific cytokines are involved in nerve-injury and inflammation-induced processes in the CNS (Zhang & An, 2007). Upon injury of the peripheral nerve tissue, macrophages and Schwan cells accumulate at the site of the injury and secrete cytokines and specific growth factors that contribute to the nerve regeneration (Erta et al., 2012). *Chemokines* are the secreted proteins that control the trafficking of selected leukocyte populations. Therefore, their chemotactic activity is described as their ability to dictate migration and activation of the leukocyte between the blood vessels and tissues. During brain development, chemokines are involved in the proliferation, differentiation, and migration of both neural and glial cells. In addition, they play a role in the maintenance of brain homeostasis. Therefore, overexpression of chemokines causes various neurological disorders such as Alzheimer's disease,

multiple sclerosis, stroke, and tumor progression (Bajetto et al. 2001). Table 2 contains examples related to the CNS-related cytokines: chemokines and interleukins (Arnoux & Audinat, 2015; Ding et al., 2015; Fontaine et al., 2008; Kang et al., 2018; Perez-Asensio et al., 2013; Tang et al., 2020).

Table 2. Cytokines, Chemokines & Interleukins in CNS

Cytokine	Source of antigen expression	Function	Important notes
IL-9 (33)(34)	Highly expressed in: •Astrocytes •Microglia •Oligodendrocyte progenitor cells	Anti-apoptotic	1. IL-9 is mainly expressed in neurons 2. IL-9 controls programmed cell death (PCD), which is a central event during **brain development**, in the murine neocortex and inhibits apoptosis. 3. IL-9 anti apoptotic effects predominantly on superficial cortical layers 4. IL-9 is important for T cell activation and differentiation in the central nervous system (CNS) autoimmune disease
IL-10 (35)	Glia cells	•Resolve inflammation •Promote wound repair at peripheral sites	1. IL-10 acts as a growth factor on SVZ progenitors and regulates neurogenesis in **normal adult brain**. 2. IL-10 is important during infection or inflammatory brain disorders. 3. In neurons, IL-10 receptor signaling has been associated with increased cellular survival and the regulation of adult neurogenesis.
KC (36)	Glial cells	Mediate leukocyte migration into injured nerve	1. KC is the predominant chemokine produced by various glial cell types after infection with TMEV 2. It is known to be associated with infiltration of neutrophils and other inflammatory cells that are believed to contribute to the pathogenesis of TMEV induced demyelinating disease in the CNS 3. KC is also involved in oligodendrocyte migration and proliferation
6CKine/ Exodus-2 CCL21	Injured dorsal root ganglion cells of the Spinal cord	Pain cascade	1.Constitutive expression of the chemokine CCL21 in the CNS leads to the development of a severe neuropathological condition and death in the majority of the animals. 2.Expression of the T cell chemoattractant chemokine CCL21 in the CNS led to the development of a striking neurological phenotype and premature death.
TIMP-1 (38)		•Neuroprotective in newborns •Potential biomarker for perinatal brain injury	1.TIMP1 deficiency exacerbated BBB disruption in a focal cerebral ischemia mode. 2.TIMP1 has a protective effect on vascular integrity in BBB. 3.TIMPs are usually use in predicting neonatal encephalopathy(NE) damage in new-borns 4.TIMPs are involved in multiple processes during development and in adulthood (3–5), including perinatal hypoxia–ischemia (HI) and white matter damage in human neonates. 5.In the adult rat central nervous system, elevated TIMP-1 expression levels may play a neuroprotective role.
Fractalkine (37)	Microglial cells	Determines several functions of microglial cells during normal CNS development	1.Recent evidence indicates that they can support survival of developing neurons, control axon outgrowth, and positioning of subsets of interneurons in the forebrain. 2.Fractalkine signaling controls the functions of microglial cells in part by regulating their timely recruitment at sites of **developing synapses**. 3.Fractalkine signaling pathway is also a key player in neurodevelopmental disorders. 4.In the CNS, fractalkine is mostly expressed by neurons and its unique receptor CX3CR1is exclusively expressed by microglia. 5.This synaptic pruning by microglia has been also observed in the hippocampus where this developmental process is regulated by fractalkine/CX3CR1signaling

1.3 TRANSFORMING GROWTH FACTOR BETA (TGF-B) SUPER FAMILY

The transforming growth factor-beta (TGF- β) superfamily are a group of both homodimers and heterodimers secreted pleiotropic regulatory proteins comprised of more than 30 members that based on their structural features, subdivided into two prominent families: (a) the TGF-β/Activin and (b) the Bone Morphogenetic Protein (BMP)/Growth and Differentiation Factor (GDF) (Hata & Chen, 2016; Lin et al., 2006; Zi et al., 2012). These families based on sequence similarities further subdivided into subfamilies including (a) TGF-β, (b) Activin, Inhibin, Nodal, and Lefty and (c) BMP, GDF, and Mülllerian Inhibiting Substance (MIS) (Hata & Chen, 2016; Kashima & Hata, 2018; Lin et al., 2006; K. Luo, 2017; Zi et al., 2012). Although there is a structural homology between these proteins, they act via specific and separate receptors in temporal and tissue-specific patterns that lead to the regulation of various non-overlapping biological responses at the transcriptional level (Kashima & Hata, 2018; Lin et al., 2006; K. Luo, 2017). Members of the TFG-β superfamily play essential roles during embryonic development, postnatal growth and adult tissue homeostasis that impose their effects by regulating cell proliferation, differentiation, motility/migration, maturation, survival/apoptosis, morphogenesis, and wound healing (Hata & Chen, 2016; Lin et al., 2006; K. Luo, 2017). Secreted TGF-β ligand upon binding to a conserved family of cell surface serine/threonine-specific protein kinase receptors recruit and activate SMAD signaling cascades through phosphorylating SMAD family transcription factors (Lin et al., 2006; Zi et al., 2012). The activation of downstream signaling molecules in various cell types causing these factors to act as growth factors, cytokines, and morphogens (based on their abilities to induce phenotype transformation) (Zi, Chapnick, and Liu 2012).

1.3.1 TGF-β_{1-3} isoforms formation, processing & activation

Among different TGF-β subfamilies, transforming growth factor (TGF)-β_{1-3} are homodimeric secreted regulatory proteins producing in mammals with high amounts of expression in the central and peripheral nervous system (Hinck, 2012; Unsicker & Strelau, 2000). A separate gene produces each isoform at a different location in a long arm of chromosome 19 (19q13.1), chromosome 1 (1q41), and chromosome 14 (14q24), respectively (Poniatowski et al. 2015). All three isoforms are secreted by both glial and neuronal cells which are required for a variety of

biological behaviors such as proper development, invasion and adhesion, repair and remodeling, and extracellular matrix (ECM) formation in the CNS (Li et al., 2017; Unsicker & Strelau, 2000). Three isoforms of the TGF-β subfamily, TGF-$\beta_{1\text{-}3}$, share 70-80% homology in their peptide sequences and their 3D structures (Li, Gu, and Yi 2017). All isoforms are translated into inactive 55kDa precursor monomers called pre-pro-TGF-βs (Poniatowski et al. 2015). However, the length of pre-pro-TGF-βs is different for each isoform; TGF-β_1 is produced with a shorter peptide sequence containing 390 amino acids, while TGF-$\beta_{2\&3}$ is encoded longer each 412 amino acids (Li, Gu, and Yi 2017). The encoded pre-pro-TGF-βs contain three conserved domains including N-terminally signal peptide (SP) of 20-30 amino acids that targets the peptide for secretion, C-terminally mature TGF-β peptide of 112-114 amino acids forming the final active and mature TGF-β, and intermediate pro-sequences called latency-associated peptide (LAP) of 249 amino acids essential for correct folding of the protein (Li et al., 2017; Poniatowski et al., 2015). Through several post-translational modification steps, pre-pro-TGF-βs transform from an inactive to an active state, the mature and active TGF-β homodimer peptides (Li, Gu, and Yi 2017). At the endoplasmic reticulum (ER), the SP of the pre-pro-TGF-βs is proteolytically cleaved off, and the remaining monomers contain a LAP and mature TGF-β peptide (Lin et al., 2006; Poniatowski et al., 2015; Zi et al., 2012). Further stages involve the formation of three non-covalent disulfide bonds between cysteine residues in the LAP region of two cleaved monomers by catalyzing activity of disulfide isomerase (PDI), to form a homodimer called pro-TGF-βs (Li et al., 2017; Poniatowski et al., 2015; Unsicker & Strelau, 2000; Zi et al., 2012). The created pro-TGF-βs homodimer contains two LAP and two mature TGF- β peptides with a total molecular weight of 110 kDa (Poniatowski et al. 2015). After it is synthesized, in the Golgi apparatus pro-TGF-βs is cleaved by proteolysis activity of a paired basic amino acid cleaving enzyme (PACE) furin, which is a pro-protein convertase. Protease furin separates the LAP region from the connected mature TGF- β peptide at the target sequence between 278 and 279 amino acid residues in each monomer of the pro-TGF-βs homodimer and produces a small latent TGF-β complex (SLC) with a molecular weight of approximately 100 kDa. However, noncovalent disulfide bond connections between two LAP regions and two TGF- β peptides/chains are maintained in the SLC (Li et al., 2017; Poniatowski et al., 2015). As a result, two connected LAP regions act as a specific type of protection with chaperon-like activity for TGF-β peptides that restricts its bioavailability and prevent from its interaction with a receptor (Derynck & Budi, 2019; Poniatowski et al., 2015; Zi

et al., 2012). Through LAP dimer regions and disulfide bound, SLC covalently binds to another protein named latent TGF-β binding protein (LTBP), and together form larger complex called large latent complex (LLC) with molecular weight of 220 kDa. LTBP is the 120–160 kDa multidomain protein that contributes to LLC proper folding and assembly that finally promotes LLC secretion to the extracellular matrix (ECM) (Derynck and Budi 2019). After secretion of LLC, LTBP forms covalent bonds with other components of the ECM such as fibronectin and fibrillin microfibrils, which cause anchoring of LLC to the specific site in the ECM. Consequently, anchored LLC is stored as an inactive biological complex in the ECM (Li et al., 2017; Poniatowski et al., 2015). Further proteolytic cleavage is required to release the active TGF-β peptide from other domains of the LLC, including LTBP and LAP pro-region. Proteases cleave LTBP from the LLC, then LAP undergoes conformational changes, and all the disulfide bonds are disrupted, and finally, bioactive, and mature TGF-β peptide homodimer with the molecular weight of 25 kDa is released from the complex and bound to its specific receptors (Li et al., 2017; Lin et al., 2006; Zi et al., 2012). It is worth noting that the activated TGF-β can bind to other TGF-β family members and form heterodimers (Li et al., 2017; Lin et al., 2006). Upon binding of both TGF-β homo and heterodimers to their special receptors, TGF-β signaling cascades are activated, which regulate different biological responses (Derynck & Budi, 2019; Lin et al., 2006; Zi et al., 2012).

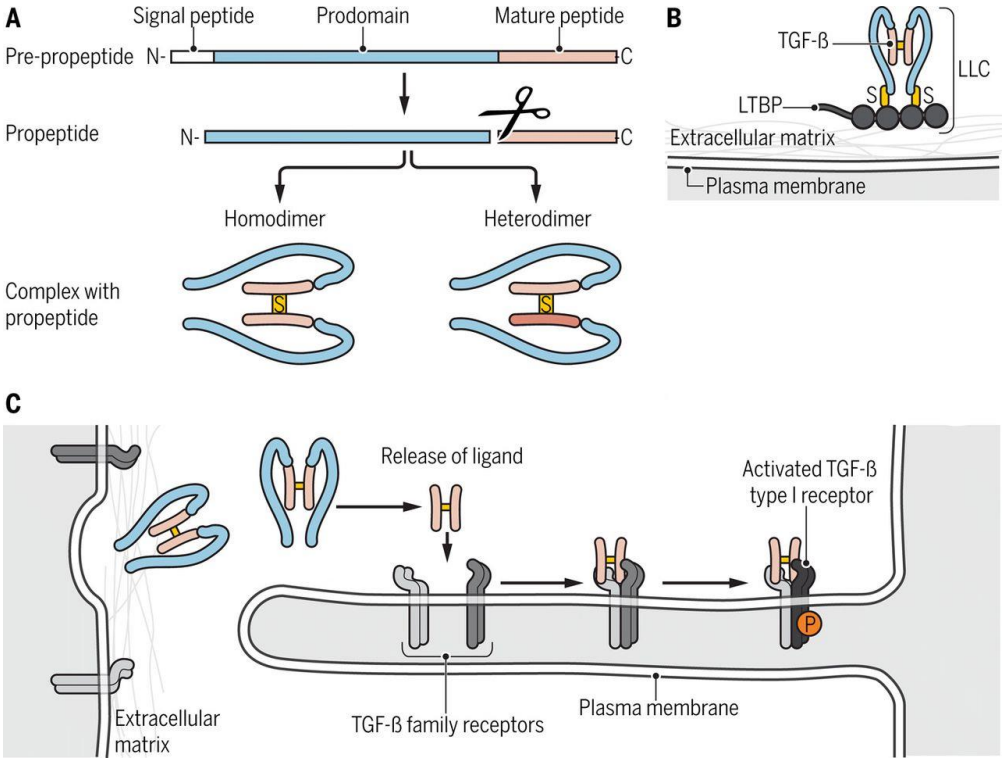


Figure 4. TGF-β Formation and presentation of the active TGF-β (Derynck and Budi 2019). **(A)** At the endoplasmic reticulum (ER), signal peptide is removed and proteolytic cleavage of the prodomain (LAP) from the mature TGF- β polypeptide is happened. In the Golgi apparatus, LAP region is separated from connected mature TGF- β peptide by protease Furin and form small latent TGF-β complex (SLC). Two LAP regions act as protection for two TGF-β peptides which are connected through noncovalent disulfide bond. **(B)** SLC covalently binds to latent TGF-β binding protein (LTBP) and together form LLC which causes anchoring of LLC to the specific site in the ECM. **(C)** LTBP dissociated from LLC with proteases and LAP undergoes conformational changes and all the disulfide bonds are disrupted. Finally, bioactive, and mature TGF-β peptide homodimer/heterodimer is released from the complex and binds to its specific receptors.

1.3.2 TGF-β signal transduction mechanism

After activation of TGF-β homodimers in the extracellular environment, which can function in a paracrine, autocrine, and Juxtacrine manner, individual parts of the TGF-β signal transduction will happen and regulate in the cytosol and nucleus of the target cell, expressing special TGF-β receptors on its surface (Li et al., 2017; Lin et al., 2006; Zi et al., 2012). There are basic key components in the TGF-β signaling pathways that play critical roles in these regulatory events. Both canonical/Smad-dependent and non-canonical/Smad-independent signaling

pathways are initiated by the binding of bioavailable dimeric TGF-βs (TGF-β_{1-3}) to type I and type II TGF-β receptor complex (TGFβRI, TGFβRII) on the surface of the target cell (Li et al., 2017; Poniatowski et al., 2015; Zi et al., 2012). Both TGFβRI and TGFβRII are a family of glycoproteins anchoring in the cell membrane with three main sections: an N-terminal small extracellular ligand-binding domain, a transmembrane part, and a C-terminal cytoplasmic region which contains serine/threonine kinase activity (Poniatowski et al., 2015; Wrana et al., 1994). TGF-β ligands binding induces dimerization of TGFβRII monomers leading to the recruitment of TGFβRI monomers (Zi, Chapnick, and Liu 2012). Together, TGF-β ligands with heteromeric receptor complex, including two of each TGFβRI and TGFβRII, form an active heterotetrameric ligand-receptor complex (Hata & Chen, 2016; Poniatowski et al., 2015; Zi et al., 2012). Both TGFβRI and TGFβRII are dual-specificity kinases with tyrosine and serine/threonine kinase activities with molecular weights of approximately 55 and 70 kDa, respectively (Hata & Chen, 2016; Li et al., 2017; Zi et al., 2012). High-affinity interaction of the TGF-β ligands directly with the TGFβRII promote phosphorylation of the two receptors on their cytoplasmic juxtamembrane regions (Hata & Chen, 2016; Kashima & Hata, 2018; Li et al., 2017; Wrana et al., 1994). Therefore, type II TGF-β receptor by its kinase activity mediate both ligand-stimulated autophosphorylation of its monomers on serine and threonine residues and transphosphorylation of the type I receptor monomers on "GS kinase domains" which are enriched in glycine and serine residues (Li et al., 2017; Wrana et al., 1994; Zi et al., 2012). Phosphorylation causes activation of TGFβRI kinase activity, leading to the phosphorylation of downstream signaling molecules and propagation of the signal into the nucleus (Li et al., 2017; Wrana et al., 1994). Activated TGF-β receptors mediate both intracellular Smad-dependent and independent signaling pathways, which all lead to the activation of various biological responses (Li, Gu, and Yi 2017). It is important to note that the bioactive TGF-βs dimers can only bind to the extracellular ligand-binding domain of the TGFβRII, and they cannot directly bind to the TGFβRI (Li et al., 2017; Poniatowski et al., 2015; Wrana et al., 1994). Consequently, autophosphorylation of TGFβRII monomers causes recruitment of TGFβRI monomers and formation of the stable heterotetrameric ligand-receptor complex (Li et al., 2017; Wrana et al., 1994).

Based on the ligand features including concentration and type (context), the target cell in which special receptors are expressed (location), and developmental stage (time) various signaling events are triggered including canonical and non-canonical pathways (Hata & Chen, 2016;

Kashima & Hata, 2018; K. Luo, 2017; Zi et al., 2012). The first step of the canonical signaling cascade is the activation of TGF-βs-receptors complex that transmit the signal into the intracellular components and regulate gene expression through the SMAD family of signal transducers (Hata & Chen, 2016; Poniatowski et al., 2015; Wrana et al., 1994; Zi et al., 2012). SMAD proteins are the nucleocytoplasmic transcription factors, with constant shuttling between cytoplasm and nucleus, that mediate intracellular signaling pathways (K. Luo, 2017; Poniatowski et al., 2015; Wrana et al., 1994; Zi et al., 2012). Upon formation of the ternary complex, TGF-βs ligand and TGFβRI/TGFβRII heterotetramer, SMAD mediators transduce activated signals to the nucleus in a context-dependence manner (Hata & Chen, 2016; Zi et al., 2012). SMAD family contains eight members that based on their different function have been classified into three distinct categories as follow; the receptor-regulated SMADs (R-Smad) including SMADs 1, 2, 3, 5, and 8, the common-partner Smad (Co-Smad) with only SMAD4, and the inhibitory Smads (I-Smad) including SMADs 6 and 7 (Li et al., 2017; Poniatowski et al., 2015). All the members of the SMAD family have almost the same structural domains including N-terminally and C-terminally located Mad homology domains 1 and 2 (MH1 & MH2) with approximately 130 and 200 amino acids, respectively. These two highly conserved domains are linked through an intervening linker region enrich in proline (Pro) (Li et al., 2017; Poniatowski et al., 2015; Zi et al., 2012). In an inactive state, both MH1 and MH2 domains of the cytoplasmic SMADs have high affinity towards each other, and consequently, they form inactivated homo-oligomers (Li et al., 2017; Zi et al., 2012). In an active state, when SMAD transcription factors translocate from the cytoplasm into the nucleus, they bind to the DNA strand and other proteins through the MH1 and MH2 domains, respectively, that cause oligomerization of Samd proteins inside the nucleus (Poniatowski et al., 2015; Zi et al., 2012). One structural exception is the I-Smads, which are made up of only one MH2 domain (Poniatowski et al. 2015).

Each member of the SMAD family is the substrate of the specific receptor kinases, naming as a “receptor-specific” R-Smads, that mediate different signaling pathways (Kashima & Hata, 2018; Li et al., 2017). Among R-smads, Smads 2 and 3 are phosphorylated by the TGFβRI and transducing signals in TGF-β signaling pathway, while rest of them (Smads 1, 5, 8) are involved in BMP signaling cascades (Hata & Chen, 2016; Li et al., 2017; Poniatowski et al., 2015). In Smad-dependent signaling pathway, after forming the TGF-β ligand-bound receptor complex and consequent phosphorylation/activation of the TGFβRI, SMAD2 and SMAD3 are recruited to the

activated TGF-β receptor complex. This translocation of the SMADs proteins from the cytoplasm to the intracellular membrane is mediated by the SMAD anchor for receptor activation (SARA) protein which binds to the phosphorylated GS domain of the TGFβRI and MH2 domains of the R-Smads (Li et al., 2017; Moustakas, 2002; Zi et al., 2012). SARA protein is the membrane-bound carrier that presents SMADs to the activated TGFβRI kinase and promotes their connection leading to the phosphorylation and activation of the R-Smads (Li et al., 2017; Moustakas, 2002; Poniatowski et al., 2015). Following phosphorylation of the R-Smads by the TGFβRI kinases and consequent conformational changes in their 3D structure, they are dissociated and released from the SARA protein and receptor complex (Li et al., 2017; Poniatowski et al., 2015; Zi et al., 2012). The phosphorylated tail of R-Smads has a high affinity to MH2 domain of the Co-Smad/Smad4, which is anchored to the cytoplasm by scaffolding proteins such as heat shock protein TGF-b type I receptor-associated protein-1 (TRAP-1) (Li et al., 2017; Moustakas, 2002; Zi et al., 2012). Together, two phosphorylated R-Smads (Smad2 &3) with one Co-Smad (Smad4) form heteromeric complexes that translocate from the cytoplasm into the nucleus (Kashima & Hata, 2018; K. Luo, 2017; Moustakas, 2002; Poniatowski et al., 2015; Zi et al., 2012). In the nucleus, the R-Smad/Co-Smad complexes through their MH1 domains bind to sequence-specific DNA-binding cofactors known as the Smad-binding elements (SBE), located in promoters of target genes, and regulate the transcription process (Kashima & Hata, 2018; Li et al., 2017; K. Luo, 2017; Moustakas, 2002).

In addition to the positive regulation, Smad-dependent signaling cascades can be negatively regulated by the antagonistic activity of inhibitory Smads (I-Smads) such as SMAD6 and SMAD7 (Hata & Chen, 2016; Kashima & Hata, 2018; Li et al., 2017; K. Luo, 2017). The I-Smads are the negative regulators of the signals which are mediated by the R-Smad/Co-Smad complexes (Hata & Chen, 2016; Poniatowski et al., 2015). These signals are inhibited at both cytoplasmic and nuclear levels throughout the signaling cascade. At the cytoplasmic level, the SMAD7, which is made up of only conserved MH2 domain, by binding to the TGFβRI blocks the recruitment of the R-Smads and interferes with the phosphorylation/activation of SMAD2/3 in a competitive manner (Li et al., 2017; Moustakas, 2002; Poniatowski et al., 2015). In addition, SMAD7 inhibits the formation of the R-Smad/Co-Smad complexes by interaction with receptor-phosphorylated R-Smads (Hata and Chen 2016). At the nuclear level, SMAD7 by interacting with the R-Smad/Co-Smad complex inhibits further interactions with the SBE and DNA (Hata & Chen,

2016; Li et al., 2017). Lastly, E3 ubiquitin-protein ligase Smurfs, also known as Smad-specific E3 ligase family, are enzymes that negatively regulate the TGF-β signaling pathway. Upon stimulation of the TGF-β-receptor complex, nuclear Smurf2 binds to the SMAD7 and exports from the nucleus to the cytosol. Consequently, Smad7-Smurf2 complex interacts with the TGFβRI and R-Smads (Smad2/3) and induces their proteasomal degradation (Hata & Chen, 2016; Koganti et al., 2018; Moustakas, 2002). It is important to note that both non-phosphorylated cytoplasmic R-Smads and dissociated nuclear R-Smads from the R-Smads/Co-Smad complex during nucleocytoplasmic shuttling of the Smads, can be targeted by the Smurfs which leads to the proteasomal degradation of R-Smads and general inhibition of the pathway (Moustakas, 2002; Zi et al., 2012). Among I-Smads, SMAD6 mainly participates in negative regulation of the BMP signaling pathway; however, it can partially block TGF-β signaling cascade (Hata & Chen, 2016; Li et al., 2017).

Stimulation of the TGF-β receptor complex can also transmit the signal to the nucleus via other intracellular signaling pathways called non-canonical or Smad-independent signaling (Poniatowski et al., 2015; Zi et al., 2012). These pathways are categorized into three main pathways, each with various branches, including mitogen-activated protein kinases (MAPK) pathway (ERK1/ERK2, Jun-N terminal kinase (JNK) and p38 and PI3K kinases), growth and survival-promoting pathway AKT/PKB, and small GTP-binding proteins (Rho-GTPase) pathway (Ras, RhoA, Rac1, CDC42, and mTOR) (Kubiczkova et al., 2012; Poniatowski et al., 2015). In addition, TGF-βs indirectly modulate other intracellular signaling pathways through extensive crosstalk activities which lead to different morphogenic events including proliferation, differentiation, migration, apoptosis, epithelial to mesenchymal transition, and matrix formation (Diniz et al., 2014; Kubiczkova et al., 2012; K. Luo, 2017; Nakashima et al., 2018; Yi et al., 2010).

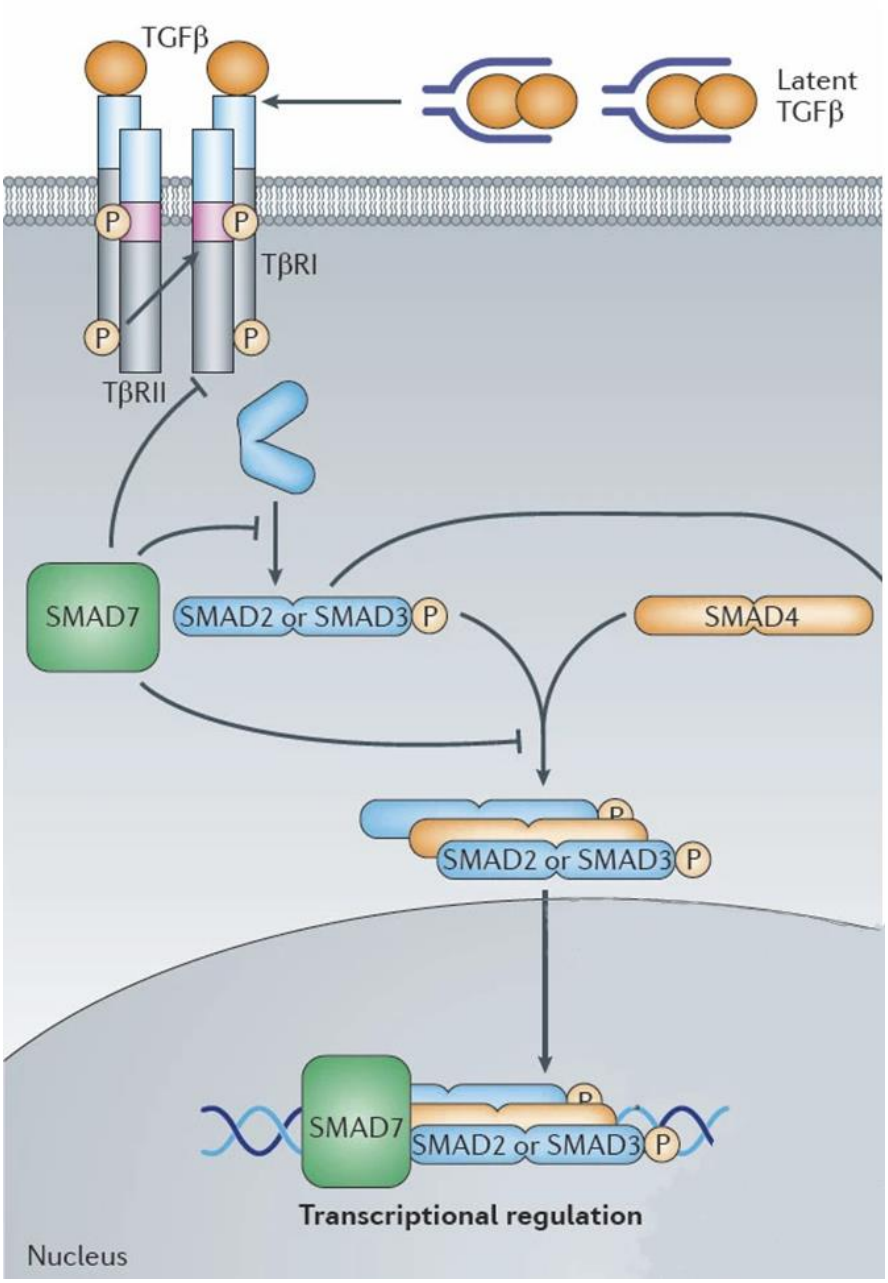


Figure 5. Canonical/Smad-dependent TGF-β signaling pathway (Akhurst and Hata 2012). Upon binding of the bioactive TGF-β ligands to the type II receptor, formation of the heteromeric complex is induced between type I and type II receptors. Type II receptors by transphosphorylation of the type I receptors cause phosphorylation and activation of the SMAD family of transcription factors, SMAD 2/3. Phosphorylated SMAD2/3 together with SMAD4 form heteromeric complexes which translocate from the cytoplasm into the nucleus where it can bind to the genomic DNA. This pathway can be inhibited at both cytoplasm and nucleus through binding to the inhibitory SMAD, SMAD7.

1.3.3 Roles of TGF-βs in CNS Development

Members of the TGF-β superfamily act in a spatiotemporal manner and play critical roles during development, adult tissue homeostasis, and scar formation/injury repair (Kashima & Hata, 2018; Nakashima et al., 2018; Wrana et al., 1994). These growth factors based on the cell type, developmental stage/time, and tissue location produce various biological outcomes (K. Luo, 2017; Zi et al., 2012). During embryogenesis and establishment of the CNS basic plan, especially the neural tube formation, TGF-β members act as graded positional cues known as morphogens (Wrana et al., 1994; Zi et al., 2012). Therefore, TGF-βs by forming long-range concentration gradients provide positional information for different progenitor cells, leading to the cell fate determination and tissue patterning (Zi, Chapnick, and Liu 2012). Morphogens signal in a dose-dependent manner, in which different fields of cells in a restricted region of the tissue accurately read the concentration of the bioavailable signaling molecules, trigger various intracellular signaling pathways, and orchestrate different biological events (Asano et al., 2009; Zi et al., 2012). One of the best examples is the process of Dorsal/Ventral patterning of the neural tube in which BMP and Shh are the dorsal and ventral morphogenic factors, which are more abundant in the roof and floor plate of the neural tube, respectively. BMP concentration decreases toward the ventral part and promotes dorsal phenotype or dorsalization. Conversely, Shh concentration decreases towards the roof plate and promotes ventralization of the neural tube (E. A. Meyers and Kessler 2017).

It has been reported that neuronal polarity is achieved during neuronal development of the mammalian brain by various intracellular signaling cascades. *In vitro* and *in vivo* studies on developing neurons demonstrated that TGF-β signaling pathways by both endogenous and exogenous ligands initiate the rapid growth of the neuronal processes, including multiple dendrites and single axon, and direct axon specification determines the fate of naïve neurites. These transition from an unpolarized to a polarized state during embryonic development is also dependent on the expression of type II TGF-β receptor (TGFβRII) over the surface of the axons; genetic ablation and genetic enhancement of the TGFβRII activity in the developing mouse neocortex neurons lead to the complete lack of axon and formation of multiple axons, respectively (Barnes & Polleux, 2009; Yi et al., 2010). These data provided evidence that number of the axon processes is determined by the level of TGFβRII activity in newborn neurons (Yi et al. 2010). In

addition, TGF-βs play essential roles in the appropriate innervation of the growing axons during development (Kashima and Hata 2018). In recent years, a number of studies have elucidated that mutation in TGF-βs ligands and receptors have been contributed to the number of human developmental disorders which are manifested by age-related deterioration of the neurons, mental retardation, and significant reduction in cognitive ability (Asano et al., 2009; Yi et al., 2010). In addition, TGF-β isoforms have compensatory effects for each other during brain development, meaning that in the complete lack of either of these isoforms, the development of the brain is normal. However, individuals with two knocked down TGF-β isoforms show significant neural developmental defects. This indicated that neural development of each cell type is controlled by the effects of different number of TGF-β isoforms and maintenance of mature neurons is dependent on specific TGF-β isoforms. For example, mice with haploinsufficiency for *Tgfb2* experience loss of specific parts of the NS and exhibit Parkinson-like defects (Asano et al. 2009).

Another critical role of TGF-βs in CNS is the growth-promoting effect on damaged neurons. Therefore, by inducing axonal growth and regeneration following CNS lesioning, TGF-β isoforms play critical roles in the survival of neurons within damaged neural tissue during the neuronal repair process (Abe et al., 1996; Li et al., 2017; Stegmüller et al., 2008). expression levels of TGF-β isoforms, especially TGF-$\beta_{1\&2}$ and their receptors, increase significantly in neural tissues after brain injuries (Stegmüller et al. 2008). Abe k et al., demonstrated that exogenous application of TGF-β isoforms to the cultured Wistar rats hippocampal neurons derived from 18-day-old embryos, after mechanical lesioning of the axons by laser beam irradiation, caused axonal regrowth and regeneration of the injured site in a concentration-dependent manner (Abe et al. 1996). In addition, levels of the TGF-β ligands and receptors are regulated in an age-dependent manner. It has been reported that astrocytes secreted TGF-βs have higher expression levels in old rats compared with postnatal or young rats (Kashima and Hata 2018). Under pathological conditions of age-related neuronal disorders such as Alzheimer's disease (AD), TGFβRII, SMAD2/3 phosphorylation, and the levels of nuclear R-Smads/Co-Smad complex decrease significantly and consequently β-amyloids accumulate in the brain of AD patients. Therefore, applying exogenous TGF-β1 is one therapeutic approach that induces activation of the pathway and clearance of the β-amyloids aggregates in AD patients (Alcantara Gomes et al., 2005; Kashima & Hata, 2018). Also, TGF- βs has a neuroprotective role and impose their pro-survival effects on various populations of neurons (Stegmüller et al. 2008). It has been reported that TGF-$\beta_{2\&3}$ controls

the development and survival of dopaminergic neurons losing in Parkinson's disease (Kashima and Hata 2018). Compelling evidence shows that secreted TGF-β from astrocyte and Schwan cells play an essential role in regulating synaptogenesis of excitatory and inhibitory neurons in both vertebrates and invertebrates (Ana P.B. Araujo et al., 2016; Diniz et al., 2014). Confirming these findings, Diniz et al., 2012 demonstrated that increasing levels of astrocytic TGF-β_1, which is considered as a synaptogenic molecule, upregulate the formation of the cortical excitatory synapses in rodent and human (Ana P.B. Araujo et al., 2016; Diniz et al., 2017). The expression pattern of TGF-β_1 and TGFβRII in the cerebellum confirms the synaptogenic effect of TGF-β_1 on glutamatergic cerebellar neurons during development (Ana Paula Bergamo Araujo, Carpi-Santos, and Gomes 2019). Araujo et al., reported that treatment of the cultured cerebellar granule cells derived from p6 mice, which express type II TGF-β receptor, by TGF-β_1 increased the levels of P-Smad and formation of the excitatory synapses between cerebellar granule cells (Ana P.B. Araujo et al. 2016).

Interestingly, downstream elements of the TGF-βs signaling cascades are required for proper development and neurogenesis of the cerebellum. Fernandes M et al., provided evidence regarding the role of SMAD4 in the maintenance of the cerebellar URL and consequent generation of the glutamatergic neuronal progenitors during early cerebellar development. Using mutant mice with deficiency in SMAD4, URL is failed to generate glutamatergic neuronal cells, which leads to defects in cerebellar foliation/cytoarchitecture and reduction in the cerebellar size. In addition, lack of SMAD4 affects the number and distribution of the GABAergic neuronal progenitors located in the cerebellar VZ, leading to the mis-localization of the PCs (Fernandes, Antoine, and Hébert 2012).

1.3.4 Role of TGF-β in induction of Epithelial Mesenchymal Transition (EMT) during embryogenesis

An epithelial-mesenchymal transition (EMT) is a biological process that occurs during both normal and abnormal physiological and pathological events (Kalluri & Weinberg, 2009; Kim et al., 2017). EMT plays essential roles ranging from embryonic development and cellular differentiation to tissue repair. However, EMT can also cause organ fibrosis, tumor formation and

malignant transformation which is known as metastasis (Kim et al., 2017; Thiery et al., 2009). During EMT, polarized epithelial cells with specialized cell-cell contacts and interactions with the basement membrane completely lose their epithelial characteristics and gain mesenchymal features such as migratory and invasiveness behaviors (Kalluri & Weinberg, 2009; Kim et al., 2017; Jian Xu et al., 2009). Therefore, cells with new mesenchymal phenotypes can migrate from their epithelial nest to near or faraway locations (Jian Xu, Lamouille, and Derynck 2009). Subsequently, cells become differentiated during development or initiate metastasis in malignancies (Thiery et al. 2009). EMT is a reversible transition by a reverse process called mesenchymal to epithelial transition (MET) (Thiery et al., 2009; Jian Xu et al., 2009). During embryogenesis, development, and disease pathogenesis, cells undergo several rounds of EMT to MET and sequentially transit between epithelial and mesenchymal states referring to as primary, secondary, and tertiary EMT (Kalluri & Weinberg, 2009; Kim et al., 2017; Thiery et al., 2009). The earliest and key EMT process occurs during gastrulation, which is known as the formation of the three distinctive embryonic germ layers including ectoderm, mesoderm, and endoderm from the epiblast (Kim et al. 2017). After gastrulation, the neural crest formation at the junction between the neural plate (neuroectoderm) and non-neural ectoderm is the other key EMT during embryonic development (Thiery et al. 2009). Consequently, neural crest cells undergo classical EMT and their phenotype switch from epithelial into migratory mesenchymal (Kalluri and Weinberg 2009). Then, individual neural crest cells migrate and disperse to form the vertebrate head and the ganglia of the peripheral nervous system (Kalluri & Weinberg, 2009; Kim et al., 2017; Thiery et al., 2009). Acquisition of the migratory mesenchymal phenotype is a hallmark of the EMT process (Kim et al. 2017). Therefore, evaluating changes in expression of epithelial and mesenchymal cell markers is commonly used to detect cells that go through the EMT process (Kalluri and Weinberg 2009).

In general, Cadherins are a large family of Ca^{2+}-dependent transmembrane glycoproteins containing more than 100 members, which are categorized into classical cadherin, desmosomal cadherin, protocadherin, and cadherin-related protein families (Paulson et al. 2014). Cadherins as the cell adhesion molecules participate in various morphogenetic events during development such as cell-cell adhesion and cell migration (Taneyhill 2008). Based on their sequence similarity, classical cadherins have been identified to be grouped into types I and II (Hiraga et al., 2020; Paulson et al., 2014). Classical cadherins are single pass transmembrane proteins with an extracellular domain containing five repeat sequences, and two cytoplasmic catenin-binding

domains that link cadherin protein to the actin cytoskeleton meshwork and intracellular signaling pathways (Kwiatkowski et al., 2007; Paulson et al., 2014; Rogers et al., 2018). All these components contribute to the rigid cell-cell adhesion mediated by the cadherins (Hiraga et al. 2020).

Epithelial cells are tightly connected to their neighbors and apicobasal axis through cell adhesion molecules such as E-Cadherin (Thiery et al. 2009). Therefore, E-Cadherin is an epithelial marker that involves in maintaining the epithelial layer (Kim et al. 2017). Conversely, mesenchymal cells lose their attachment capacity and gain migratory and invasive properties (Kim et al., 2017; Thiery et al., 2009). N-Cadherin is a mesenchymal marker whose expression makes the cell more motile (Jian Xu, Lamouille, and Derynck 2009). Thus, during an EMT and conversion of epithelial cells to mesenchymal cells, expression of E-Cadherin needs to be repressed which leads to downregulation of E-Cadherin accompanied by upregulation of N-Cadherin (Kalluri & Weinberg, 2009; Taneyhill, 2008; Thiery et al., 2009). Therefore, the loss of *E-Cadherin* gene expression, which causes epithelial cells to lose their connections and transit into migratory mesenchymal cells, with increased expression of *N-Cadherin* gene are the important hallmarks that show the passage of a cell and EMT (Kim et al., 2017; Jian Xu et al., 2009).

The EMT process is triggered and regulated by various extracellular ligands such as growth and differentiation factors, mediators and different signaling pathways. All these environmental signals by regulating (downregulation/upregulation) the expression of epithelial and mesenchymal genes, which are involved in epithelial cells polarity, cell-cell adhesion, migration, and invasiveness, contribute to the activation of EMT process (Kalluri & Weinberg, 2009; Kim et al., 2017; Jian Xu et al., 2009). During early embryonic development and in normal/transformed cell lines, different conserved signaling pathways activate the EMT process's activation (Kim et al. 2017). Several studies have demonstrated that TGF-β signaling pathway play an important role in induction and regulation of the EMT during embryogenesis (Kalluri & Weinberg, 2009; Kim et al., 2017). It has been reported that treatment of cultured epithelial cells with TGF-β causes activation of TGF-β signaling pathway, in which activated SMAD proteins regulate transcription of three families of transcription factors including Snail, ZEB and bHLH families (Jian Xu, Lamouille, and Derynck 2009). Consequently, proliferation of the epithelial cells and expression of the epithelial markers are repressed, and mesenchymal markers expression enhanced, leading

to the increased motility of the cells (Kim et al., 2017; Jian Xu et al., 2009). Therefore, TGF-β by reprograming the expression of Cadherin genes causes phenotypic changes in the cells (Kalluri & Weinberg, 2009; Kim et al., 2017; Taneyhill, 2008; Jian Xu et al., 2009).

1.3.5 Role of TGF-β_1 in induction of Autophagy

Autophagy is a tightly regulated lysosomal degradation and clearance pathway (Fleming & Rubinsztein, 2020; Lee et al., 2015; Mcknight et al., 2012). This conserved intracellular catabolic pathway by trafficking cytoplasmic contents to the lysosome and degrading them supply energy during normal and abnormal physiological conditions (Fleming & Rubinsztein, 2020; Lee et al., 2015; Mizushima & Levine, 2010). As a cell self-digestive process, autophagy is highly active during differentiation and development (Mcknight et al., 2012; Mizushima & Levine, 2010). During embryogenesis, autophagy, by responding to extracellular signals, contributes to rapid cellular changes necessary for development (Mizushima and Levine 2010). In addition, autophagy plays an essential role in maintaining cellular homeostasis by degrading damaged organelles and unfolded, aggregated, aggregate-prone, and long-lived proteins (Damme et al. 2015a). Therefore, basal autophagy by mobilizing energy within the cell environment continuously and without a specific stimulus happens as a self-renewal mechanism to overcome or compensate stress conditions such as starvation (Damme et al., 2015; Mizushima & Levine, 2010). Based on the delivery method of the materials to the lysosome, autophagy is divided into three subtypes: macroautophagy, microautophagy, and chaperon-mediated autophagy (Mcknight et al., 2012). Macroautophagy is an evolutionarily conserved mechanism which is characterized by the formation, fusion, and degradation of double-membrane vesicle containing cytoplasmic components called autophagic vacuole (AV) or autophagosome (Fleming & Rubinsztein, 2020; Lee et al., 2015; Mcknight et al., 2012). Formation of the autophagosome requires the expression of the ATG proteins that associate with its membranes such as ATG12-ATG5-ATG16L1 complex and LC3β (ATG8) (Fleming & Rubinsztein, 2020; Lee et al., 2015). During autophagosome formation, LC3β is cleaved by ATG4 and form LC3β -I. Then, LC3β-I conjugate to the lipid phosphatidylethanolamine (PE) in autophagosome membranes; this conjugated form is called LC3β-II (Fleming & Rubinsztein, 2020; Mcknight et al., 2012). Lipidation of LC3β, conversion of LC3β-I to LC3β-II, is an important step playing a critical role in the expansion, closure, fusion,

and degradation of the autophagosome which is commonly used as a hallmark of the activated autophagy (Fleming & Rubinsztein, 2020; Lee et al., 2015).

In postmitotic and terminally differentiated cells like neurons, which are not able to attenuate the detrimental aggregates by cell division, autophagy contributes to their health and homeostasis by eliminating cytoplasmic contents (Damme et al., 2015; Fleming & Rubinsztein, 2020; Mcknight et al., 2012; Mizushima & Levine, 2010). The specialized and polarized structure of the neurons makes them sensitive to aggregated cytosolic proteins or organelles, which act as a stress for cells. Therefore, basal autophagy is continuously activated at a low level and without starvation signals in the CNS, which describes the neuroprotective role of autophagy in neurons (Mcknight et al., 2012). In addition, basal autophagy in neurons by degrading misfolded and unfolded proteins generates amino acids and macromolecules necessary for new protein synthesis. This turnover of the materials keeps the equilibrium between catabolic and anabolic pathways and maintains the neural homeostasis under healthy conditions (Damme et al., 2015; Fleming & Rubinsztein, 2020; Mcknight et al., 2012). It has been shown that autophagosome production is increased after physical or chemical injury of the axon, which were seen at soma and axon terminals of the injured neuron. In addition, brain electron microscopy (EM) images of the postmortem patients with neurodegenerative diseases showed large number of aggregated autophagosomes in neurons. These data support the idea that autophagy plays critical roles during axonal outgrowth and neural development (Mcknight et al., 2012).

Different studies have reported that TGF-β_1 through SMAD signaling pathway induces simultaneous autophagy in various cell models such as HuH7 human hepatocellular carcinoma cells (Suzuki, Kiyono, and Miyazono 2010) and non-small cell lung cancer cell lines (NSCLC) A549 and H1975 (Alizadeh et al. 2018). TGF-β signaling by upregulating autophagy-related genes and their mRNA expression levels enhances autophagy flux through Smad and non-Smad pathways (Kiyono et al., 2009; Suzuki et al., 2010). Upon binding of TGF-β to TGFβRI and II and activating the SMAD family of transcription factors, transcription of autophagy-related genes such as ATG proteins increased. Consequently, autophagosomes accumulate in the cells which are detected by conversion of microtubule-associated protein 1 light chain 3 (LC3β) to the lipidated form, LC3β-II (Alizadeh et al., 2018; Kiyono et al., 2009; Suzuki et al., 2010). Therefore, TGF-β, by increasing the rate of LC3-II turnover, enhances the degradation rate of

cytoplasmic contents (Suzuki, Kiyono, and Miyazono 2010). It has been reported that TGF-β-induced autophagy attenuated in knockdown of SMAD2/3 and SMAD4 (Kiyono et al. 2009). Therefore, TGFβ$_1$ signaling is involved in a wide range of cellular functions such as autophagy which protects cells from various stress conditions, including nutrient deprivation, growth factor depletion, and hypoxia (Kiyono et al., 2009; Suzuki et al., 2010).

1.3.6 Distribution of TGF-B ligands & receptors in the developing CNS

The three TGF-β isoforms are primarily expressed in the mammalian central and peripheral nervous system by both glial and neuronal cells (Alcantara Gomes et al., 2005; Asano et al., 2009; Unsicker & Strelau, 2000; Yi et al., 2010). *In vivo*, each isoform has a different and unique cellular distribution which characterize its functionality (Asano et al., 2009; Unsicker & Strelau, 2000). Consequently, mutations in TGF-β isoforms lead to defects from partial to complete malformation and/or malfunction of the CNS to lethal conditions (Asano et al. 2009). Expression of TGF-β isoforms in neuroepithelium is recognized during the neural tube formation at earliest embryonic day E8.5 (Yi et al. 2010).

Early immunohistochemical studies showed that TGF-β$_1$ does not have widespread localization in the intact brain and its expression is restricted to choroid plexus epithelial and meningeal cells, while strongly expressed in other cell types including neurons, astrocytes, and microglia upon brain lesioning (Alcantara Gomes, de Oliveira Sousa, and Romão 2005). However, further studies demonstrated that during the formation of the cerebellar primordium, TGF-β$_1$ is expressed by cells in the proliferative zones including EGL and the cerebellar nuclei neurons (Ana P.B. Araujo et al., 2016; Mecha et al., 2008). *In vitro* studies on primary cultured neurons derived from different regions of the mouse brain including embryonic cerebral cortex and midbrain, and newborn cerebellum, demonstrated that TGF-β$_1$ predominantly presented in neural soma of the midbrain neurons and through the neural processes of the cerebellar and cortical neurons in a punctate arrangement (Alcantara Gomes et al., 2005; De Oliveira Sousa et al., 2004). Immunohistochemistry and PCR assay results from cultured cerebellar granule cells showed that both TGFβRII and TGF-β$_1$ protein and mRNA are distributed throughout the GCs, which make them either a target or source for TGF-β$_1$ (Ana P.B. Araujo et al. 2016). In addition, the expression

pattern of TGF-β_1 and its receptors in migrating neurons of the developing cortex and radial glia support the idea that TGF-β_1 may participate in neural migration (Alcantara Gomes et al., 2005; Ana P.B. Araujo et al., 2016; Yi et al., 2010).

TGF-$\beta_{2\&3}$ have widespread localization throughout the developing CNS especially in radial glial cells and neuronal cell types in the telencephalic cortex, hippocampus, brain stem and spinal cord (Alcantara Gomes et al., 2005; Unsicker & Strelau, 2000). In addition, there are supporting evidence that TGF-$\beta_{2\&3}$ play important roles in neural migration and differentiation (Alcantara Gomes et al., 2005). Several studies reported that TGF-$\beta_{2\&3}$ and their receptors are co-expressed during early embryonic days in ventral domains of the neural tube including notochord and floor plate as well as developing dopaminergic neurons of the midbrain (Alcantara Gomes et al., 2005; Yi et al., 2010). Therefore, the expression of TGF-β2&3 by ventral midbrain dopaminergic neurons makes them potent survival factors that can be used as therapeutic agents for Parkinson's disease (Alcantara Gomes et al., 2005; Roussa & Krieglstein, 2004). In the adult rat CNS, TGF-$\beta_{2\&3}$ is expressed almost in all parts including the cerebral cortex, hippocampus, brainstem, and cerebellum (Alcantara Gomes et al., 2005). Similarly, TGFβRI and TGFβRII are presented in both developing and adult NS regions including the cerebral cortex, midbrain, cerebellum, and brain stem with higher levels of expression by migrating neurons and radial glia in a spatiotemporal manner (Alcantara Gomes et al., 2005; Ana P.B. Araujo et al., 2016; De Oliveira Sousa et al., 2004; Vivien et al., 1998). Sagittal sections through the P6 mouse cerebellum have revealed the distribution of the TGFβRI and TGFβRII among three different cellular layers; PCL and ML which are stained with anti-calbindin and anti-β-tubulin III show colocalization with the marker of the TGF-β receptors (Ana P.B. Araujo et al., 2016; Ana Paula Bergamo Araujo et al., 2019). *In situ* hybridization study showed TGFβRII mRNA expression in the rat hind brain at embryonic day E14 (Alcantara Gomes et al., 2005). Expression of downstream elements of the TGF-β signaling pathway, including the SMAD family of proteins, is characterized in different developing cerebellum regions (Fernandes et al., 2012; Stegmüller et al., 2008). SMAD2 and 3 are widely presented in ML and PCL located in developing rat cerebellar cortex which are phosphorylated in URL and VZ (Stegmüller et al. 2008). Conversely, SMAD4 is strongly expressed in the GL of the embryonic cerebellum (Fernandes, Antoine, and Hébert 2012).

1.4 RATIONAL OF THE STUDY

The cerebellum is critical for motor control and cognitive function. Structural changes in neuronal connectivity characterize many cerebellar neurological disorders. The cerebellar functional process occurs through a complex circuit assembled around input via mossy and climbing fibers and output through the cerebellar nuclei (CN). This is not clear how the complex cerebellar circuitry is assembled during development, and how the cellular and molecular mechanisms underlying the early cerebellar connectivity are established.

The establishment of precise cerebellar circuit connectivity involves several stages, including neurogenesis, cell migration, axonal growth and guidance, and synapse formation (Cioni et al. 2013). These stages, which start during the embryonic period and protract to postnatal days, are tightly regulated by various signaling molecules expressed in a spatiotemporal manner (Cioni et al., 2013; Saywell et al., 2014). Therefore, coordinated expression of specific signaling molecules and their related receptors at specific developmental stages leads to neurogenesis, neuronal migration, axonal and dendritic growth, branching, and synaptogenesis, which establish neuronal connectivity (Saywell et al., 2014).

The roles of TGF-β signaling pathways have been frequently reported in different stages of CNS development from neurogenesis and cell fate determination to proliferation, differentiation, migration, and survival/apoptosis of the neural cells (Alcantara Gomes et al., 2005; Ana P.B. Araujo et al., 2016; Nakashima et al., 2018). Additional evidence for critical functions of this cytokine is provided by the widespread expression of TGF-β isoforms and their receptors throughout the CNS (Ana P.B. Araujo et al. 2016). Researchers have identified that among three TGF-β isoforms, TGF-β_1 is present in the proliferative zones of the cerebellar primordium during development (Ana P.B. Araujo et al., 2016; De Oliveira Sousa et al., 2004). The cerebellar primordium emerges at approximately embryonic day (E) 7–8 from the rostral lip of the fourth ventricle in the mouse (D Goldowitz, 1998; Marzban et al., 2015; SOTELO, 2004; Wang & Zoghbi, 2001). Coordinated production of the multiple neural cell types within the cerebellar primordium is a critical step during the development of the cerebellum (Fernandes et al., 2012). The cerebellar primordium contains two distinct germinative zones: the ventrally located ventricular zone and dorsally-located rhombic lip (Englund 2006). All GABAergic neurons such as PCs are derived from the ventricular zone between E10-E13, starting at around E10.5, followed

by differentiation and formation of the PCs plate before E14.5. While all the glutamatergic neurons, including GCPs, are derived from the rhombic lip between E9-E12 (Fernandes et al., 2012; Wang et al., 2005; Wang & Zoghbi, 2001). Projection neurons of the CN have a dual origin, including RL-derived glutamatergic CN neurons born between E9 to E12 with the peak at around E11.5 and VZ-derived GABAergic after E12 (Fernandes et al., 2012; Wang et al., 2005). PC and CN neuron are the only neurons exist at around E9-E13 in the cerebellar primordium. Different studies and analyses on wild-type and mutant mice have shown the distribution and function of the TGF-β_1 and other key components of the TGF-β signaling pathways in the developing cerebellum. It has been reported that the expression of the TGF-β_1 in the EGL and the NTZ containing CN neurons may contribute to the differentiation of the cerebellar primordium (Stegmüller et al. 2008). In addition, immunohistochemistry studies revealed the high expression levels of the SMAD4 and phosphorylated SMAD2, downstream mediators of the TGF-β signaling pathway, in two main germinal zones of the cerebellar primordium including URL and VZ between E8.5 to E13 (Fernandes et al., 2012). The presence of the TGF-β_1 and SMAD family of proteins in the germinal zones of the cerebellar primordium supports the idea that this cytokine may regulate the specification and cell fate determination of the neural subtypes and their localization in the cerebellar primordium during development (Fernandes et al., 2012). In addition, mutant mice with a deficit in either of TGF-β_1 and its downstream effectors, including SMAD2, 3, and 4, showed increased neural apoptosis and death and a significant reduction in PCs dendritic arborization, foliation, and cerebellar size (Alcantara Gomes et al., 2005; Ana P.B. Araujo et al., 2016).

Based on the various studies and what we currently know about the development of the cerebellum during the earliest embryonic days, it is necessary to understand the mechanism that underlies the formation of distinct domains within the cerebellar primordium. These domains contain postmitotic cells, including CN neurons and PCs that form NTZ and PCs clusters in PCP. However, there are a couple of unanswered questions: What factors contribute to the CN neurons and PCs neurogenesis during the early stage of embryonic development? How are these factors regulate based on the time and place that they are expressed? How do neural populations survive during the earliest embryonic days? Moreover, what are the crosstalk and cooperation between signaling molecule-receptor sets and the outcome of the signaling pathway, which is mainly unknown.

One possible answer for these questions can be cooperation between intracellular signaling cascades activated at the same developmental stage. Upon binding the TGF-β ligands and activating the TGF-β signaling through phosphorylation of SMAD2/3, crosstalk with other signaling pathways can occur, leading to different cellular responses (K. Luo, 2017; Zi et al., 2012). These crosstalk activities play essential roles in regulating different biological responses during early cerebellar development (K. Luo 2017). However, it remains largely unknown how TGF-β_1 by regulating biological processes such as cell-cell adhesion and autophagy could form the neural domains within the cerebellar primordium. One approach that may contribute to forming the neural populations/domains is cadherins, which could provide multiple robust and weak interconnections between cells. On the other hand, autophagy could act as a protective process and contribute to neural survival and homeostasis during early cerebellar development. Also, autophagy is induced in response to different stresses such as starvation and hypoxia in postmitotic neurons, which cannot be regenerated (Damme et al., 2015; Mcknight et al., 2012; Nikoletopoulou et al., 2015). Several studies reported the significant role of TGF-β_1 in regulating both cadherins expression during the EMT process and autophagy at postnatal days. However, there is a big gap in investigating the role of TGF-β_1 in cerebellar development during the early embryonic period.

In the present study, using mouse embryonic cerebellar tissue, we discuss about the molecular mechanisms underlying the precise and temporal regulation of the intracellular signaling network that form through crosstalk of the TGF-β signaling pathway with other signaling pathways, including Cadherins-dependent cell-cell adhesion and Autophagy at earliest embryonic days at E9-E13. Our study will help illustrate the role of TGF-β1 during early cerebellar development and may answer the questions about neural connectivity and survival of postmitotic cerebellar neurons.

1.5 HYPOTHESIS & OBJECTIVES

1.5.1 Hypothesis

Therefore, based on the solid evidence related to the role of TGF-β_1 in CNS patterning and development, we hypothesis that TGF-β_1 may control early cerebellar development by modulating the levels of cell adhesion molecules (CAMs) and autophagy pathway in the mouse cerebellar primordium. In this regard, our study would aim to examine the expression of key

proteins participating in neural connectivity and autophagy pathway. In addition, to show that TGF-β_1 can induce changes in cadherins and autophagy flux which altogether contributes to the formation of the cerebellar domains during early cerebellar circuit formation.

1.5.2 Objectives

Using RT-qPCR, Western blot, and ISH data derived from Allen DMBA, we aimed to:

Specific aim# 1: Illustrate the activation of the Canonical TGF-β signaling pathway by looking at its downstream signaling molecules.

Specific aim# 2: Investigate the expression of CAMs and autophagy flux in cerebellar primordium from E9 to E13.

Specific aim#3: Study the effect of TGF-β1 on the expression levels of the downstream mediators of the TGF-β signaling (SMAD2/3), Cadherins, and autophagy-related proteins during early cerebellar development from E9 to E13.

2 CHAPTER II: MATERIAL & METHODS

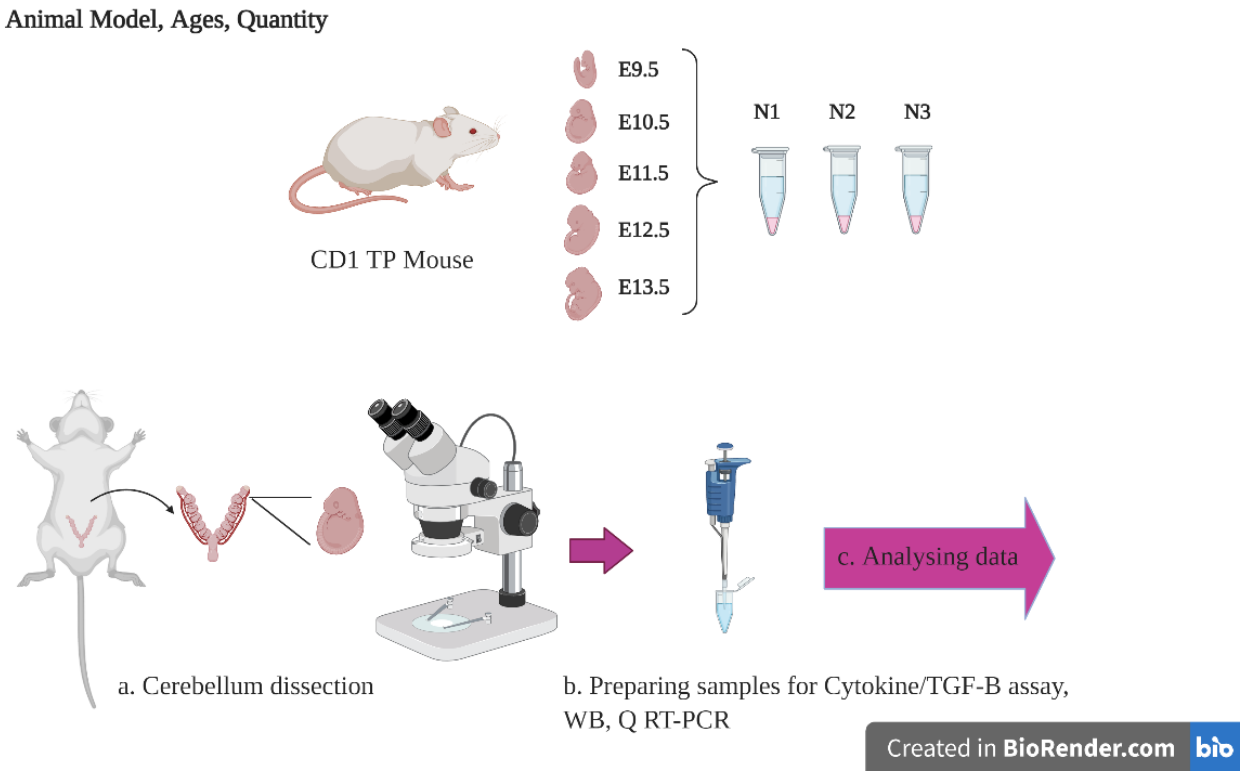
2.1 ANIMAL MAINTENANCE

All animal procedures conformed to institutional regulations and the *Guide to the care and use of experimental animals* from the Canadian Council on Animal Care (CCAC) which has been approved (protocol# AC11527) by the Bannatyne campus Animal Care Committee (ACC), University of Manitoba whose mandate it is to minimize the number and the discomfort experienced by laboratory animals used in experimental procedures. Animals were obtained from the central animal care services, faculty of Medicine, University of Manitoba, Winnipeg, MB, Canada.

2.2 SAMPLE COLLECTION

In our study, we used timed-pregnant CD1 female mice at gestation days 9.5 to 13.5, which 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. Embryos at selected embryonic days were removed from the uterus of impregnated mouse using a set of fine forceps and placed in petri dish containing ice-cold 0.1 M phosphate buffer saline (pH 7.4). Consequently, muscular wall of the uterus, Reichert's membrane and visceral yolk sac were separated, and embryos removed. Using SteREO microscope (Discovery.V8, Carl Zeiss) and tweezers with tapered tips with high precision points (Excelta™ 5SA), ectoderm and meninge were carefully removed. Finally, cerebellar primordium was detached from the surrounding tissues. The harvested tissue pieces submerged in RNAlater-ICE Stabilization Solution (Ambion, Catalog# AM7030) and lysis buffer composed of NP-40 lysis buffer (150 mM Sodium Chloride, 1.0% NP-40, 50 mM Tris PH 8.0), protease inhibitor cocktail (Life Science, No.cat# M250) and phosphatase inhibitor (Sigma Aldrich, No.cat# P5726) to protect cellular RNA and proteins, respectively. Collected samples maintained at -80°C before processing. Each replicate of samples aggregated with a minimum of 100 CD1 embryos.

Figure 6. Animal model and sample collection



2.3 MULTIPLEX CYTOKINE ASSAY

The collected embryonic cerebellar tissues covered with lysis buffer, almost double the volume of the tissue, were homogenized by sonication, and centrifuged at 12,000 x g for 10 minutes at 4°C. Then supernatant was transferred to a new tube. Protein concentrations were assessed using a commercial BSA kit (Bio-Rad No.5000121). To determine the optimal dilution and as a proof of concept, pilot assay was performed using 0.4 µg/µl, 1 µg/µl, and 2µg/µl dilutions of each sample obtained from different embryonic days. According to the manufacturer's instruction (Eve Technologies, Calgary, Canada), samples were diluted in standard PBS (pH 7.4) as the dilution buffer and stored at -80°C before sending to Eve Tech. A 150 µl aliquot of each age

was supplied for Multiplex LASER bead Discovery assays for total of 57 chemokines and 3 different isoforms of TGF-βs as follow:

Table 3. Mouse Cytokine/ Chemokine assays performed on Cerebellar Tissue Homogenates

Mouse Cytokine/ Chemokine Assays	Cytokines
Mouse Cytokine Array / Chemokine Array 44-Plex (MD44)	Eotaxin, Erythropoietin, 6Ckine, Fractalkine, G-CSF, GM-CSF, IFNB1, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17, IL-20, IP-10, KC, LIF, LIX, MCP-1, MCP-5, M-CSF, MDC, MIG, MIP-1α, MIP-1β, MIP-2, MIP-3α, MIP-3B, RANTES, TARC, TIMP-1, TNFα, and VEGF
Mouse Cytokine Array / Chemokine Array 13-Plex (MD13)	EPO, 6Ckine, Fractalkine, IFNB1, IL-11, IL-16, IL-20, MDC, MCP-5, MIP-3a, MIP-3B, TARC, TIMP-1
Cytokine Array, TGF-β 3-Plex (Multi-Species) (TGFB1-3)	TGF-beta 1, 2, and 3

Each assay was performed in duplicate. Cytokine level values were analyzed, and final concentration of 1 µg/µl was selected as the optimum dilution for all the samples. All the assays were repeated over three different replicates for each embryonic day. Data were analysed using one-way ANOVA ($P<0.05$).

2.4 IMMUNOBLOTTING

The protein analysis of the cerebellar homogenates derived from CD1 mouse embryos at embryonic days 9.5 to 13.5 was carried out using a conventional protocol (Bailey et al. 2013)(Mannan et al. 2004). Isolated cerebellar tissues covered with lysis buffer almost double the

volume of the tissue were homogenized by sonication and centrifuged at 12,000 x g for 10 minutes at 4°C. Protein concentration of the supernatant was measured by BSA kit (Bio RAD laboratories, USA; cat no. 500-0114). Then, loading samples were prepared by adding the loading buffer (Tris-Hcl 60mM, glycerol 25%, SDS 2%, mercaptoethanol (ME) 14.4Mm, bromophenol blue 0.1%, H2O) to an appropriate volume of the sample to have the final concentration of 1µg/µl.

Western blot samples were heated for 10 min at 90°C. After cooling down, equal amounts of protein (20 µg) were separated by a polyacrylamide gel based on the molecular weight of the proteins using 4% stacking gel and 10-15% running gels. Ten micro-liter of precision plus protein all blue standards were used as marker standard (Bio RAD laboratories, USA; cat no. 161-0373) and loaded parallel to experimental samples. Then, Proteins were transferred to PVDF membrane (Millipore, Mississauga, ON, USA) in transfer buffer (500nM glycine, 50mM tris-HCl, and 20% methanol) at RT for 2 hours and voltage 100V.

After blocking the membranes with 5% skim milk (non-fat dried milk in TBST 0.2% (1X TBS + 0.2% Tween 20) at RT for 1 h, membranes were probed with appropriate primary antibodies (table 2) in 1% skim milk in TBST 0.2% overnight at 4ºC with gentle agitation. Membranes were then washed 3 times for 10 min in TBST 0.2% at room temperature, then incubated for 2 h at RT with horseradish peroxidase (HRP) conjugated secondary antibodies (table 3) in 1% skim milk in TBST 0.2%. Blots were washed 3 times for 10 min in TBST 0.2%. Afterwards, the membranes were soaked in the enhanced chemiluminescence (ECL) reagents (1ml Peroxide Reagent and 1ml Luminol/Enhancer) (Catalog No. 170506; Clarity Western ECL Substrate) for 60 sec. Finally, binding was detected by the BIORAD ChemiDoc Imager and quantified using the dosimetry software Alpha Ease FC.

Table 4. Primary antibodies used for Immunoblotting

Name of Antibody	Dilution	Source
Anti- N- Cadherin (CDH2)	1:500	Developmental Studies Hybridoma Bank Catalog# MNCD2
Anti- NCAM	1:500	Developmental Studies Hybridoma Bank Catalog# 5B8
Anti-CDH8	1:1000	Developmental Studies Hybridoma Bank Catalog# CAD8-1
Anti-βcatenin	1:2000	BD Bioscience Catalog# 610153
Anti- Smad 2/3	1:1000	Cell Signaling Technology Catalog# 5678S
Anti- Psmad 2	1:1000	Cell Signaling Technology Catalog# 18338S
Anti- P62	1:3000	Cell Signaling Technology Catalog# 3912S
Anti- LC3β- I/II	1:3000	Sigma-Aldrich Catalog#L8918

Table 5. Secondary antibodies used for Immunoblotting

Name of Antibody	Dilution	Source
HRP conjugated goat anti-rat IgG	1:5000	Millipore, AP136P
HRP conjugated goat anti-mouse IgG	1:5000	Millipore, AP308P
HRP conjugated goat anti-rabbit IgG	1:5000	Millipore, AP307P

2.5 RNA EXTRACTION & cDNA SYNTHESIS

The harvested cerebellar tissue pieces from mouse embryos at embryonic days 9.5 to 13.5 immediately submerged in RNAlater-ICE Stabilization Solution for overnight at 4°C, then removed from reagent and stored at -80°C. Total RNA was isolated and purified using RNeasy Plus Mini Kit (QIAGEN: Cat Nos: 74134 & 74136) followed according to the instruction of the kit. Optimal RNA yield and purity was determined by Nano-Drop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized by a qScript cDNA Synthesis kit (Quanat-bio, Catalog number: 95048-100) using 1000 pg of RNA.

2.6 REAL-TIME PCR

Quantitative reverse transcriptase-PCR was performed in a final volume of 20 μL containing 2 μL of cDNA template, 1.2 μL of exon-specific primer pairs (Forward & Reverse), and 10 μL SYBR Green Master Mix (Applied Biosystems™, Catalog No. A25742) in a 96-well plate. The primers are listed in Table 4. All reactions were performed in duplicate, and β–actin was chosen as a reference gene to normalize gene expression values. Thermal cycling and quantification were performed with the real-time PCR instrument (Quantastudio3, Applied Biosystems) using the following cycling parameters: an initial denaturation at 50°C for 2 min, and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Specificity of the amplified product was determined by melt-curve analysis immediately following the completion of the final amplification cycle. The relative expression of genes was normalized to the amount of β–actin mRNA in the same cDNA by the comparative CT method. All reactions were performed in three independent experiments and data are presented as means ± SEM.

Table 6. Primer Sequences used for mRNA quantification

Gene	Forward sequence 5′ to 3′	Reverse sequence 5′ to 3′
N-Cadherin (cdh2)	CGG TTT CAC TTG AGA GCA CA	CAT ACG TCC CAG GCT TTG AT
Ncam	TGG TTC CGA GAT GGT CAG TT	GGA TGG AGA AGA CGG TGT GT
cdh8	TGC TGA CGA CCC AGT TTA TG	GGT TCC AGA CAG ACC ACC AG
βcatenin	CAG ATC CCA TCC ACG CAG TT	ATT GCA CGT GTG GCA AGT TC
TβRI	ATT GCT GGT CCA GTC TGC TT	CCT GAT CCA GAC CCT GAT GT
TβRII	ACT GTC CAC TTG CGA CAA CCA GAA	AGA AGC GGC ATC TTC CAG AGT GAA
βactin	CTG TCC CTG TAT GCC TCT G	ATG TCA CGC ACG ATT TCC

2.7 STATISTICAL ANALYSIS

All experiments were repeated 3 times per each embryonic day. For statistical analysis and figure preparations One-way and Two-way ANOVA were performed using Graphpad Prism 8.0.2 statistical software and *P*-values < 0.05 were considered significant. Results are showed as mean ± SEM.

3 CHAPTER III: RESULTS

3.1 SCREENING OF CYTOKINES/CHEMOKINES/TRANSFORMING GROWTH FACTORS IN MOUSE EMBRYONIC CEREBELLAR TISSUES AT E9-E13.

In order to evaluate the expression profile of the cytokines/chemokines/transforming growth factors during early cerebellar development, levels of a total of 57 cytokines/chemokines and three different isoforms of TGF-βs were assessed. Based on the protocol mentioned in Method and Material, cerebellar tissues of CD1 mouse embryos at embryonic days E9.5-E13.5 were collected and analyzed for cytokine and chemokine levels using Mouse Cytokine/Chemokine Array 44-plex, 13-plex, and TGF-β 3-plex (Eve Technologies, Calgary, AB, Canada). Our results showed different patterns of expression for each cytokine/chemokine. We categorized the markers based on their family. Out of 57 cytokines/chemokines and three isoforms of TGF-βs, total of 19 protein as follow: six Interleukins (IL), seven Chemokines, two Cytokines, two growth factors and two isoforms of TGF-βs are considerably expressed in the dissected cerebellar tissues at early developmental stages.

The analyzed data related to ILs demonstrated that the expression amounts of four ILs, including IL-2, IL-9, IL-10, and IL-1β, neither increased nor decreased by time and remained almost unchanged across five embryonic days, with minor alteration in IL-2 and IL-9 amounts. However, IL-10, IL-1β values were maintained at the same level from E9 to E13. All the differences by the time were not considerable (Figure 7. A). As can be seen, IL-16 and IL-15 values did not follow a specific pattern of expression over the given embryonic period. IL-16 showed rapid and significant fluctuation in its diurnal expression level. The IL-16 expression in embryonic cerebellar tissue increased sharply from E9 to E10. Then, values fell at E11 and E12, followed by a significant increase at E13. Similarly, data showed mild fluctuation in IL-15 expression profiles. However, the differences did not reach statistical significance (Figure 7. B).

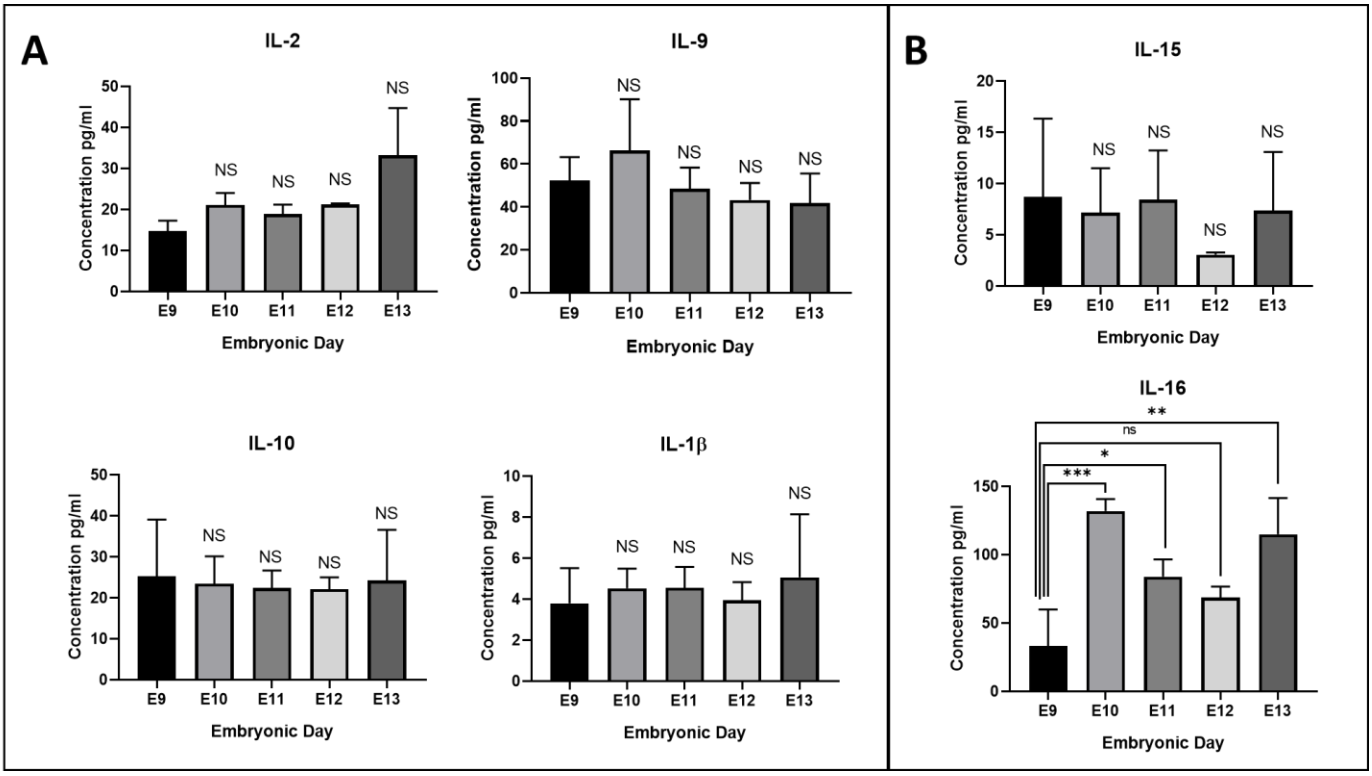
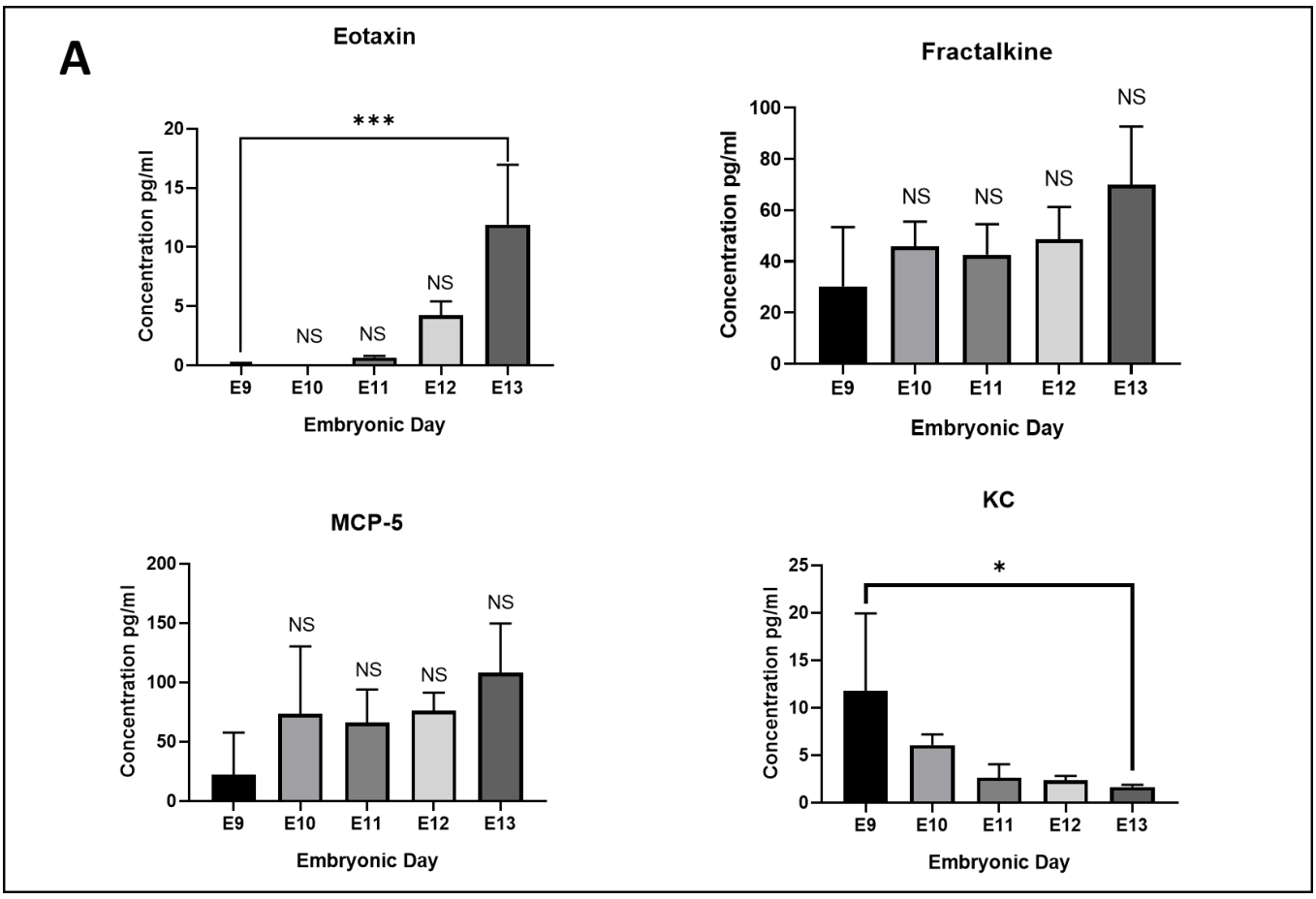


Figure 7. **The Interleukins expression profile in Cerebellar Primordium during early developmental stages at E9-E13. (A, B)** Cerebellar tissues derived from CD1 mouse embryos at E9-E13 were analyzed for different Interleukins. All the assays were repeated over three different replicates for each embryonic day (n=3). Each replicate of samples aggregated with a minimum of 100 CD1 embryos. Data were analyzed using one-way ANOVA (P-value ≤ 0.05 and lower were considered as statistically significant). **(A)** Cerebellar expression levels of IL-2, IL-9, IL-10, IL-1β remained almost constant from E9-E13 with marginal changes in IL-2 and IL-9. **(B)** IL-15 and IL-16 values continuously rise and fall over time. IL-16 expression levels experienced sharp and significant fluctuation, while IL-15 showed a mild and non-significant shift in its values.

Our results showed that chemokines including Eotaxin, Fractalkine, and Monocyte chemotactic protein 5 (MCP-5) showed an upward trend and upregulation in their expression levels from E9 to E13. Results demonstrated that Eotaxin values at E13 are significantly higher than E9. Although Fractalkine and MCP-5 expression levels increased over time, their differences between embryonic days are not statistically significant. The only chemokine with a downward trend and significant downregulation is Keratinocyte chemoattractant (KC), with higher and lower amounts of expression at E9 and E13, respectively (Figure 8. A). Analyzed data related to chemokines also

demonstrated that Monocyte chemoattractant protein-1 (MCP-1) and Macrophage Inflammatory Protein 2 (MIP-2) values remained almost unchanged across five embryonic days. In addition, Macrophage Inflammatory Protein 1α (MIP-1α) amounts did not follow a specific pattern of expression over the given embryonic period and showed mild fluctuation. All the differences by the time were not considerable (Figure 8. B).



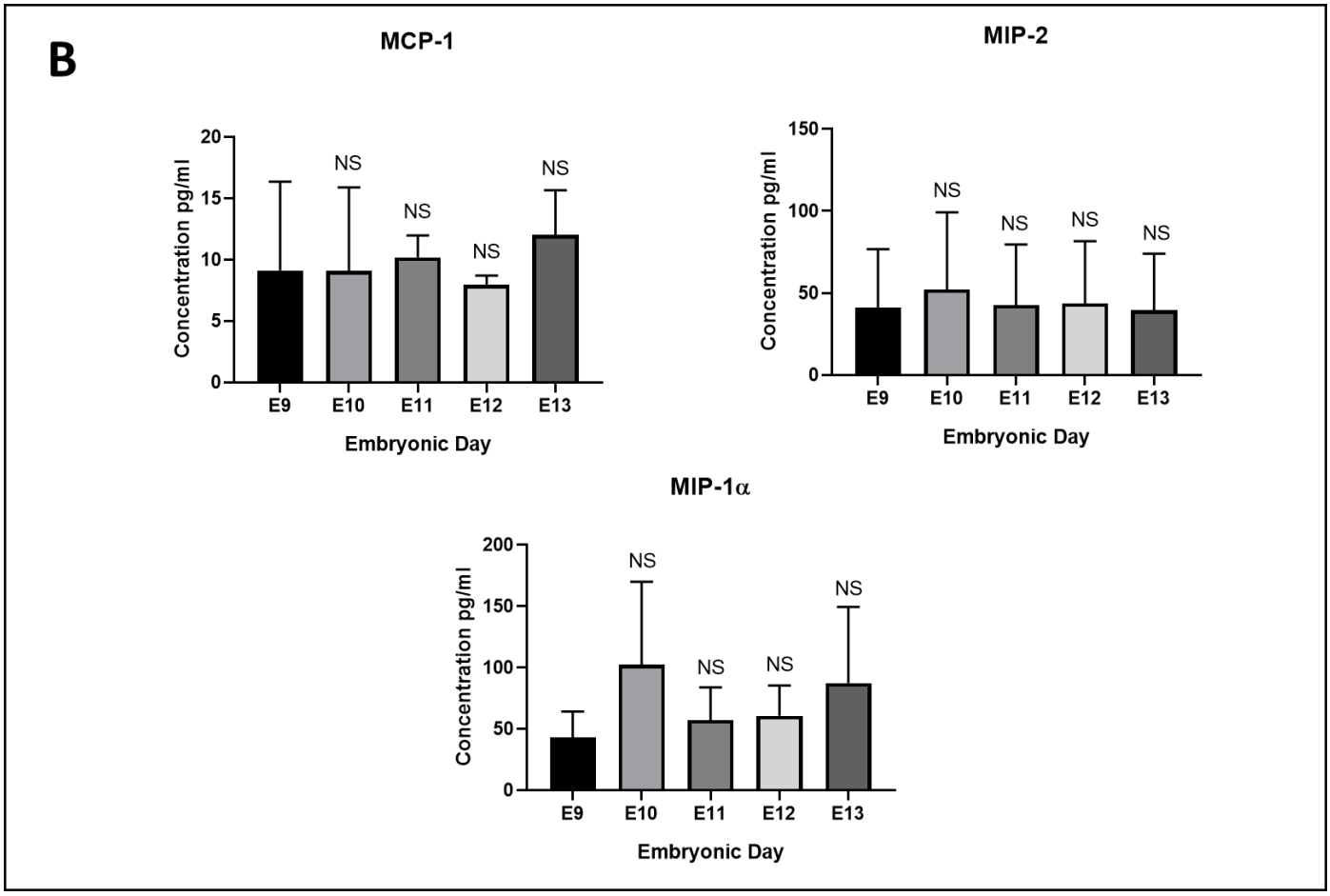


***Figure 8*. The Chemokines expression profile in Cerebellar Primordium during early developmental stages at E9-E13. (A, B)** Cerebellar tissues derived from CD1 mouse embryos at E9-E13 were analyzed for different Chemokines. All the assays were repeated over three different replicates for each embryonic day (n=3). Each replicate of samples aggregated with a minimum of 100 CD1 embryos. Data were analyzed using one-way ANOVA (P-value ≤ 0.05 and lower were considered as statistically significant). **(A)** cerebellar expression of Eotaxin increased significantly over time. Fractalkine and MCP-5 values experienced a non-significant upward trend from E9-E13. While the trend for KC is negative with a significant reduction from E9 to E13. **(B)** Cerebellar expression levels MCP-1, MIP-2 remained almost constant from E9-E13 with marginal changes in MCP-1 levels. MIP-1α values continuously rise and fall over time.

The Cytokine's expression levels demonstrated that the expression levels of 6Ckine/Exodus 2 (CCL21) acquire the highest values among all the examined cytokines/chemokines/Interleukins. The general trend for 6Ckine/Exodus 2 showed that the expression of this cytokine increased

significantly over time with the maximum level of expression at E13. In addition, interferon-gamma-induced protein 10 (IP-10) values at E13 are significantly higher than E9 (Figure 9).

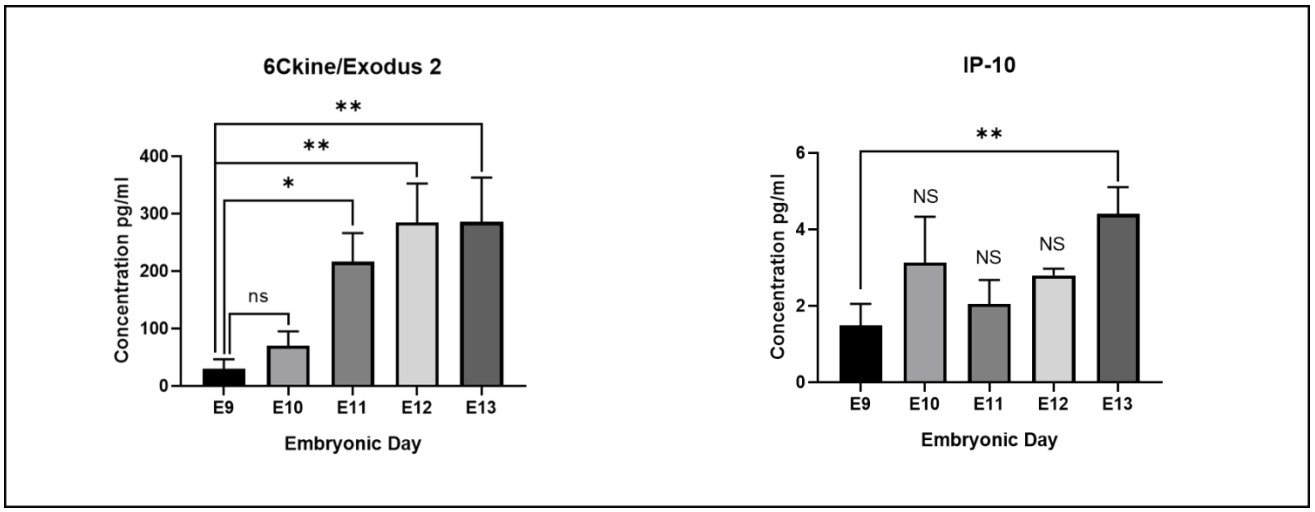


***Figure 9*. The Cytokines expression profile in Cerebellar Primordium during early developmental stages at E9-E13.** Cerebellar tissues derived from CD1 mouse embryos at E9-E13 were analyzed for different Cytokines. All the assays were repeated over three different replicates for each embryonic day (n=3). Each replicate of samples aggregated with a minimum of 100 CD1 embryos. Data were analyzed using one-way ANOVA (P-value ≤ 0.05 and lower were considered as statistically significant). 6Ckine/Exodus 2 amounts experienced a significant increase across five embryonic days and reached its peak at E13. cerebellar expression of IP-10 increased significantly over time.

According to the analyzed data, TIMP-1 expression has the highest rate. Its expression experienced dramatic growth from E9 to E10 and reached its highest level at E10. Consequently, TIMP-1 expression amounts showed a downturn and hit the lowest point at E13. The TIMP-1 values are decreased by time from E10 to E13, although there is no statistical significance between different embryonic days. In addition, expression levels of vascular endothelial growth factor (VEGF) demonstrated that its values remained almost unchanged and with minor alteration across five embryonic days. All the differences by the time were not considerable (Figure 10).

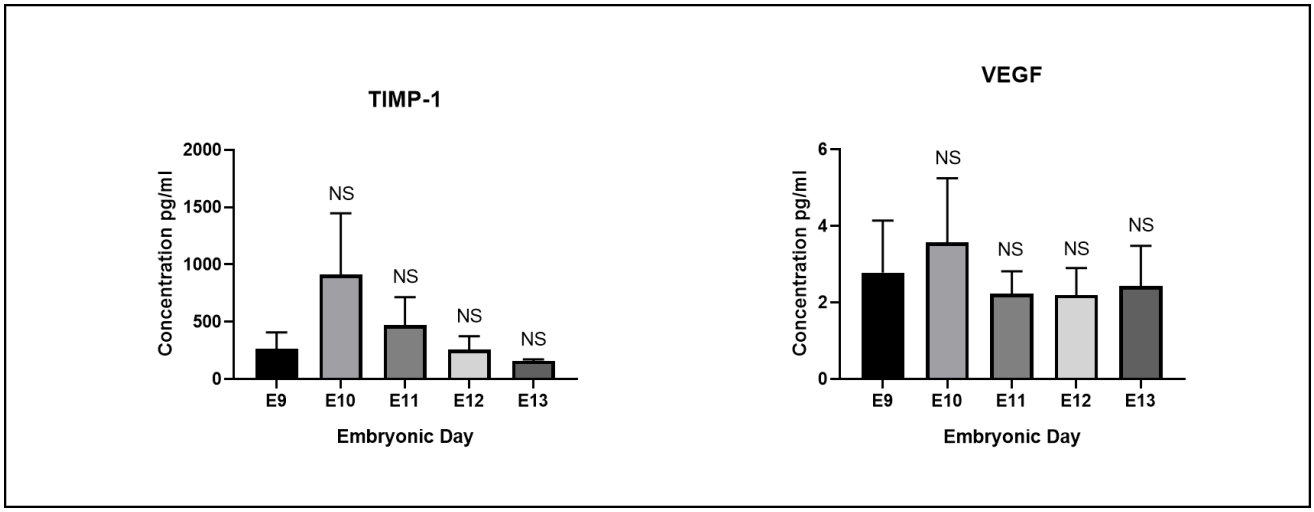


Figure 10. The TIMP-1 and VEGF expression profile in Cerebellar Primordium during early developmental stages at E9-E13. Cerebellar tissues derived from CD1 mouse embryos at E9-E13 were analyzed for different Cytokines. All the assays were repeated over three different replicates for each embryonic day (n=3). Each replicate of samples aggregated with a minimum of 100 CD1 embryos. Data were analyzed using one-way ANOVA (P-value ≤ 0.05 and lower were considered as statistically significant). TIMP-1 expression level at E10 reached the highest level among all Cytokines/Chemokines with a non-significant reduction from E10 to E13. Cerebellar expression levels of VEGF remained almost constant from E9-E13 with marginal changes in its values.

Profiling of three different isoforms of TGF-βs, TGF-β1-3, showed the presence of two isoforms (out of three) in the mouse embryonic cerebellar tissue at E9 to E13. Analyzed data demonstrated the expression of activated forms of both TGF-β1 and TGF-β2, while there was no evidence for the presence of the TGF-β3 during early cerebellar development. The most significant point based on the given data is that the expression of active TGF-β1 was intensely high at earliest embryonic days at both E9 and E10, followed by a substantial reduction in its value from E11 to E13. These data highlight the possible role of TGF-β1 during the earliest developmental stages of the cerebellar primordium, which is shown by the overexpression of this cytokine at E9 and E10. In addition, the presence of the activated TGF-β1 in the cerebellar primordium could be the sign of TGF-β signaling pathway activity during early developmental stages. According to the data, TGF-β2 expression showed almost the same trend as TGF-β1, with higher expression levels at E9 and E10 and a non-significant decline in its amount from E11 to E13. Therefore, among the three

different isoforms of the TGF-βs, TGF-β1-3, both TGF-β1&2 experienced a downward trend from E9 to E13 with the highest expression levels during the earliest developmental stages E9 and E10, followed by reduction from E11 to E13 (Figure 11).

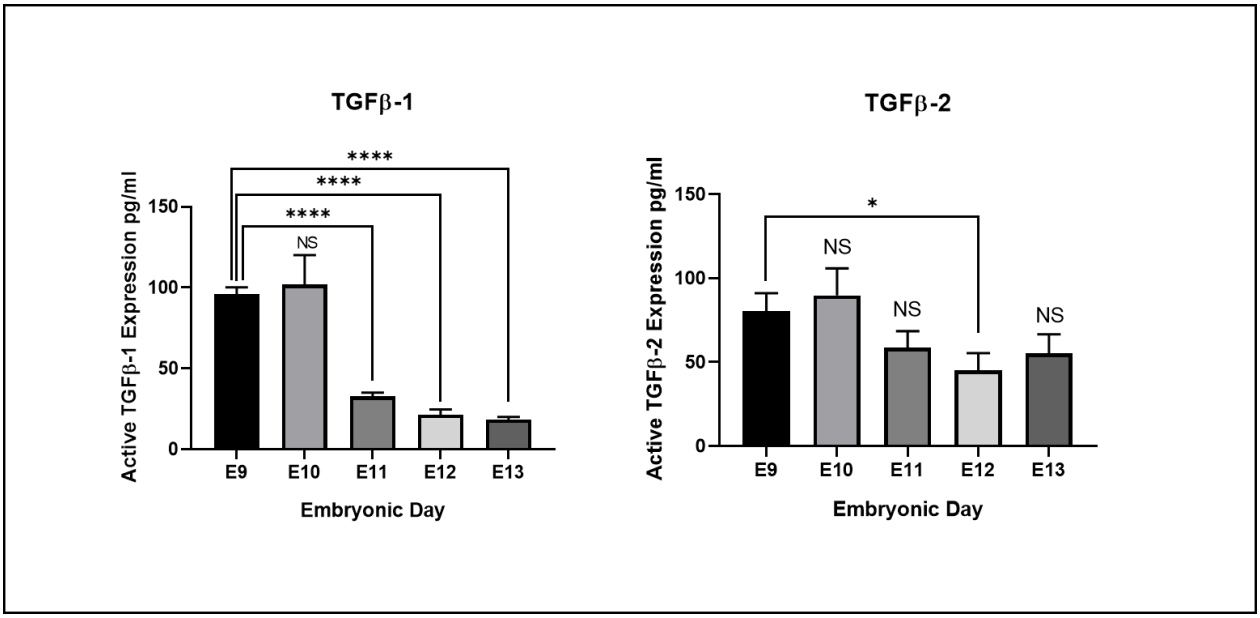


Figure 11. Transforming Growth Factor βs (TGF-β_{1-3}) expression levels in Cerebellar Primordium during early developmental stages at E9-E13. Cerebellar tissues derived from CD1 mouse embryos at E9-E13 were analyzed for different isoforms of the TGF-β_{1-3}. All the assays were repeated over three different replicates for each embryonic day (n=3). Each replicate of samples aggregated with a minimum of 100 CD1 embryos. Data were analyzed using one-way ANOVA (P-value ≤ 0.05 and lower were considered as statistically significant). TGF-β_1 highly expressed during earliest embryonic days at E9 and E10. Its expression levels decreased significantly with considerable differences from E11 to E13. TGF-β_2 values experienced almost the same expression pattern as TGF-β_1, with higher and lower amounts at E9-E10 and E11-E13, respectively. However, the changing expression pattern of TGF-β_2 is not statistically significant.

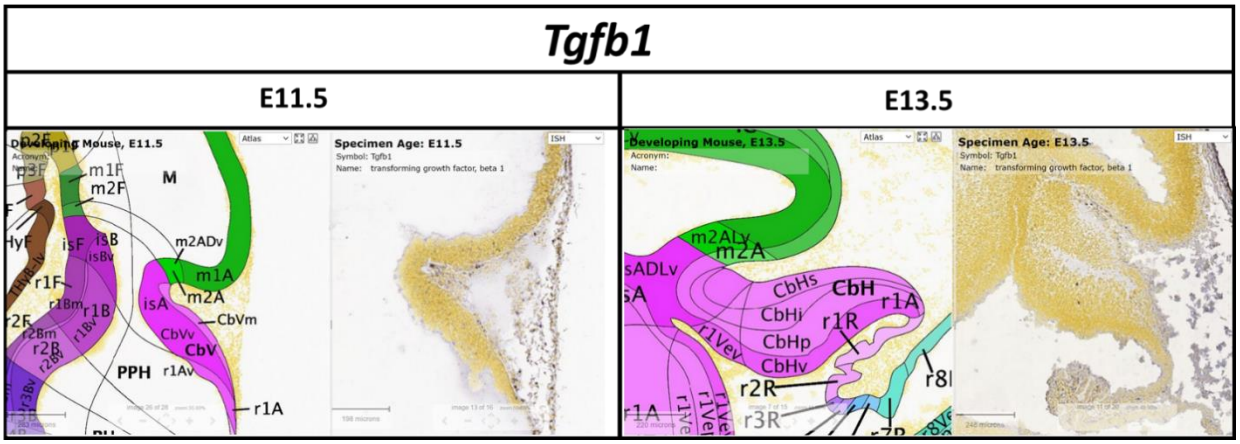
3.2 LOCALIZATION OF TGF-B_1 AND TGF-B_2 RNA SEQUENCES IN MOUSE EMBRYONIC CEREBELLAR TISSUES AT E9-E13.

In the next step, to investigate the precise localization of the *Tgfb1 and Tgfb2* RNA nucleic acid sequences and their gene expression profiling in mouse embryonic cerebellar tissue sections, we used *RNA in situ* hybridization (ISH) data using "Allen Developing Mouse Brain Atlas"

(RRID:nif-0000–00509)(http://developingmouse.brain-map.org). The Allen DMBA contains spatial and temporal genoarchitecture of the brain development that provides analyzes gene expression and localization of RNA sequences in the series of sagittal sections through the entire embryo (whole mount ISH) or brain, which were detected by RNA probes. ISH analyzed data in Allen DMBA are categorized into embryonic and postnatal mouse brain developmental time points including embryonic days (E) 11.5, E13.5, E15.5, E18.5, and postnatal days (Henry & Hohmann, 2012; Thompson et al., 2014). In our study and based on the given embryonic days (E9-E13), we used ISH analyses data at E11.5 and E13.5 to investigate the gene expression based on the anatomical regions of the cerebellar primordium.

The spatial-temporal gene expression pattern of *Tgfb1* reveals its punctate RNA expression at E11.5 in pia mater of the cerebellar primordium, tela choroidea of the of fourth ventricle. However, *Tgfb1* expression is not detected by RNA probes at E13.5 in sagittal sections through cerebellar primordium, only at low level in the neuroepithelium of the fourth ventricle (Figure 12. A). RNA expression of *Tgfb2* at both E11.5 and E13.5 time points is indistinguishable in cerebellar primordium (Figure 12. B).

A



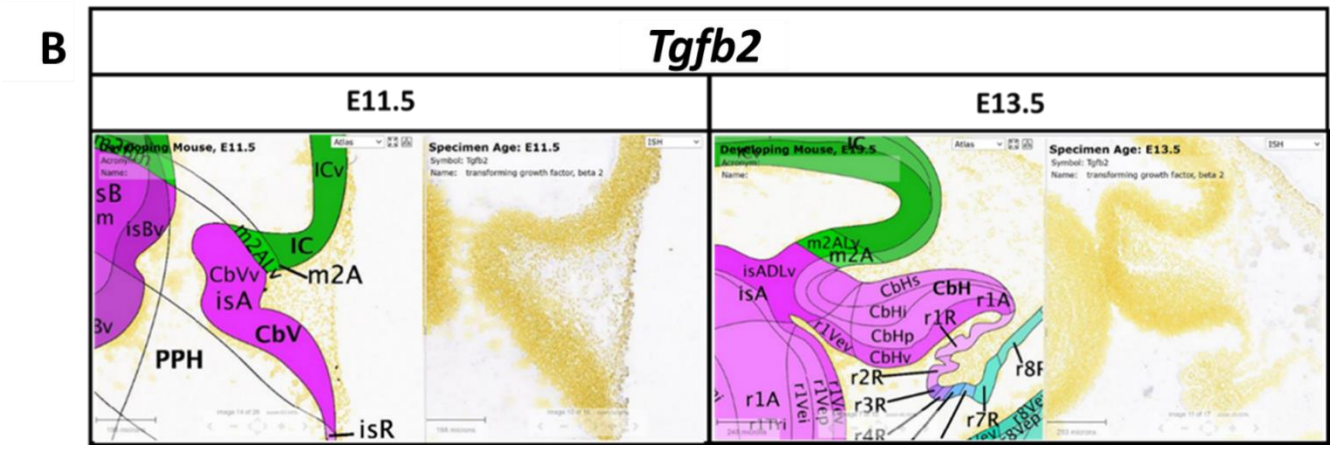


Figure 12. Allen Developing Mouse Brain Atlas ISH data of the Tgfb1 & Tgfb2 in the developing mouse cerebellum. (A, B) RNA in situ hybridization data from Allen Developing Mouse Brain Atlas was queried for **(A)** Tgfb1 (http://developingmouse.brain-map.org/experiment/show/100077823) and **(B)** Tgfb2 (http://developingmouse.brain-map.org/experiment/show/100079168) at E11.5 and E13.5. Selected images of serial sagittal sections at each time point are presented. A matched drawing atlas and ontologies (left image) show that TGF-β1 was only detected at E11.5 in pia mater of the cerebellar primordium and tela choroidea of the of fourth ventricle and very low level in the neuroepithelium. ISH patterns of TGF-β2 on sections at E11.5, and E13.5 were not detectable.

3.3 Presence of Canonical TGF-β signaling elements in mouse cerebellar tissues during earliest developmental stages at E9-E13.

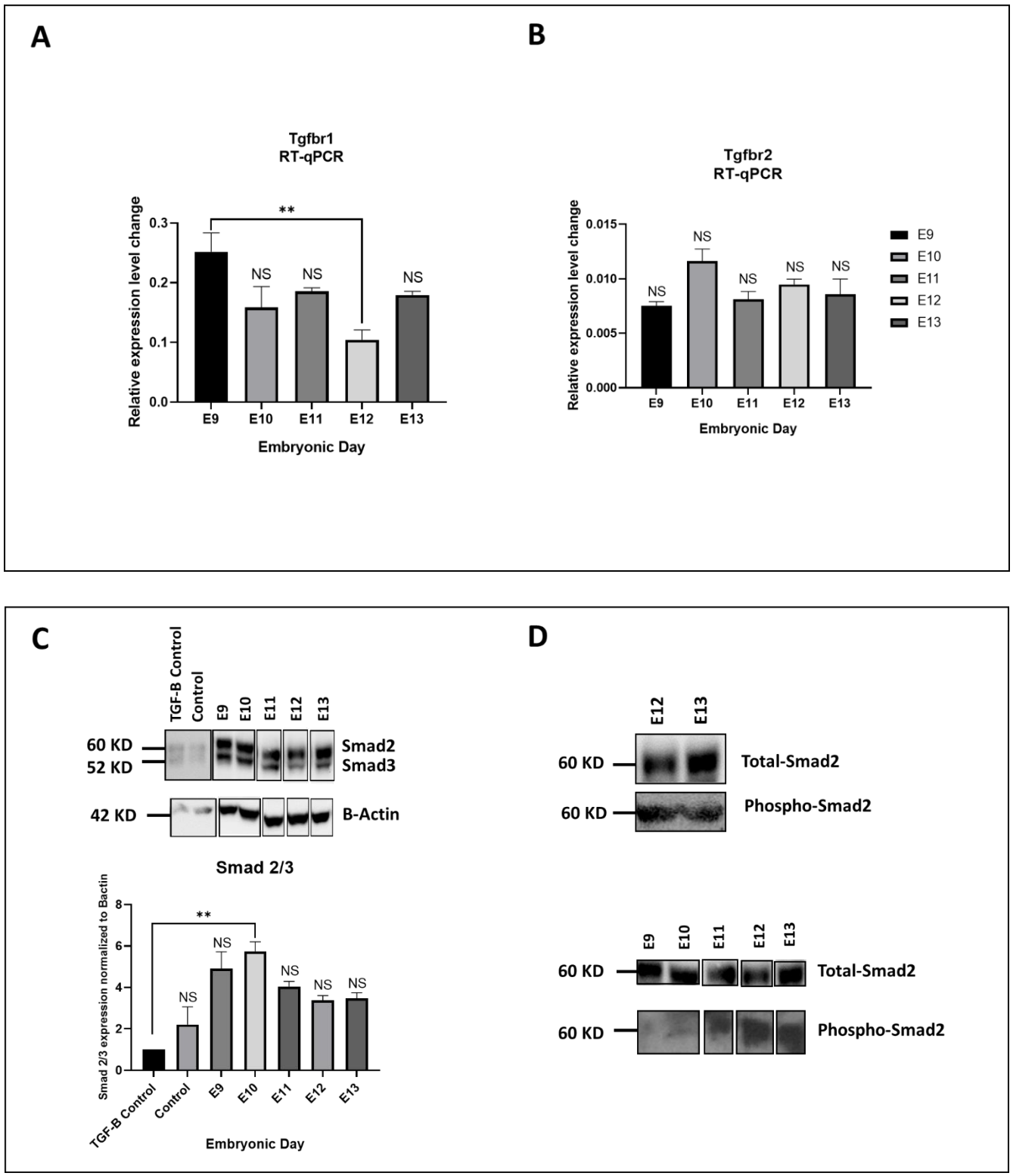
Both intracellular SMAD-dependent and independent signaling pathways are initiated by binding of the bioavailable dimeric TGF-βs (TGF-β1-3) to type I and type II TGF-β receptor complex (TGFβRI, TGFβRII) on the surface of the target cell. The first step in the canonical signaling cascade is the activation of TGF-βs-receptors complex, which transmits the signal into the intracellular components and regulates gene expression through SMAD family of signal transducers. The cerebellar expression of type 1 and type 2 TGF-β receptors (TGFβRI & II) during embryonic developmental stages is unknown, especially at earliest embryonic days from E9 to E13.

To move forward confidently, the cerebellar expression of TGFβRI and TGFβRII mRNA derived from mouse embryos at E9-E13 were analyzed with RT-qPCR. Results demonstrated that both *Tgfbr1* and *Tgfbr2* mRNA express across the given embryonic period. According to the

analyzed data, *Tgfbr1* mRNA expression is higher at embryonic day 9, which is significantly decreased at E12 (Figure 13. A). There is an almost constant level of *Tgfbr2* mRNA expression from E9 to E13, which is not statistically significant between different embryonic days (Figure 13. B).

Secreted TGF-β ligands upon binding to conserved family of cell surface serine/threonine-specific protein kinase type I and II receptors recruit and activate SMAD signaling molecules and phosphorylate them. To further clarify the connections between our data, Western blot was performed, and total SMAD 2 and 3 protein levels were evaluated in embryonic cerebellar tissues at E9-E13. We used A549 cell lines untreated (-) and treated with TGF-β_1 as controls. Western blot results showed an almost downward trend in total SMAD2 and 3 protein expression levels. Representative data reported overexpression of total SMAD2 and 3 during earliest embryonic days at E9 and E10 compared to the TGF-β_1 treated cells. In addition, there is statistical significance in total SMAD 2 and 3 protein expression amounts at E10. However, the values from E11 to E13 ultimately reduced and became even (Figure 13. C).

In addition, Western blot results showed upregulation of Phosphorylated SMAD2 from E9 to E13, with lower and higher amounts of expression at E9 and E13, respectively. Based on the given data, we found an inverse correlation between total and phosphorylated SMAD2 expression patterns, downregulation of total SMAD2, and upregulation of PSMAD2. These findings indicate the conversion of total SMAD2 to PSMAD2 and activation of the canonical TGF-β signaling pathway during early developmental stages of cerebellar primordium from E9 to E13 (Figure 13. D).


A
Tgfbr1
RT-qPCR
Relative expression level change
0.3
0.2
0.1
0.0
**
NS
NS
NS
E9
E10
E11
E12
E13
Embryonic Day
B
Tgfbr2
RT-qPCR
Relative expression level change
0.015
0.010
0.005
0.000
NS
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NS
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E9
E10
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E12
E13
Embryonic Day
C
TGF-B Control
Control
E9
E10
E11
E12
E13
60 KD
52 KD
Smad2
Smad3
42 KD
B-Actin
Smad 2/3
Smad 2/3 expression normalized to Bactin
8
6
4
2
0
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NS
NS
NS
NS
NS
TGF-B Control
Control
E9
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Embryonic Day
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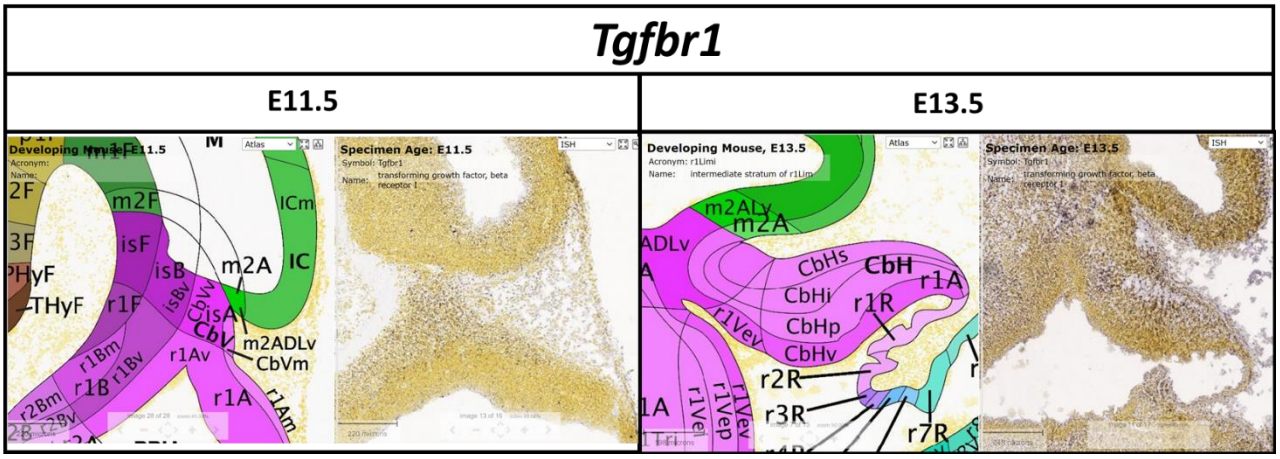
Total-Smad2
60 KD
Phospho-Smad2
E9
E10
E11
E12
E13
60 KD
Total-Smad2
60 KD
Phospho-Smad2

Figure 13. Presence of Tgfbr1 & Tgfbr2 mRNA and downregulation of total SMAD2 & 3 and upregulation of PSMAD2 in cerebellar primordium during early developmental stages at E9-E13. (A-D) Expression of Tgfbr1 and Tgfbr2 mRNA and total SMAD2 & 3 proteins in cerebellar tissues derived from CD1 mouse embryos at E9-E13; **(A, B)** mRNA expression of Tgfbr1 and Tgfbr2 were measured by RT-qPCR using extracted RNA samples from the mouse embryonic cerebellar tissues. **(A)** Tgfbr1 transcription at E9 is higher with a significant reduction at E12. **(B)** mRNA values of Tgfbr2 maintained at the same level with no significant changes from E9- E13. This experiment was repeated over three replicates for each embryonic day (n=3). **(C)** Total SMAD2 & 3 and **(D)** phosphorylated SMAD2 protein expression was assessed by Western blotting. Total SMAD 2 and 3 values upregulated at E9 and E10, compared to their correspondent controls, with a non-significant reduction from E11 to E13. Phosphorylated SMAD2 experienced an upward trend from E9 to E13. The blots were quantified compared to the TGF-β_1 treated cells using densitometry software Alpha Ease FC. The protein loading was confirmed using B-actin. The blots are representative of 3 different independent experiments. The data in the bar graphs are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA (P-value ≤ 0.05 and lower were considered statistically significant).

Expression of downstream signaling elements of the canonical TGF-β signaling pathway including type I and 2 TGF-β receptors, and SMAD2 and 3 transcription factors at E11.5 and E13.5 time points were supported by the Allen DMBA. In situ hybridization patterns for *Tgfbr1* and *Tgfbr2* in sagittal sections during cerebellar development showed dynamic expression of *Tgfbr1* at both E11.5 and E13.5. Moderate expression of *Tgfbr1* is detected in nuclear transitory zone (NTZ) at E11.5 (Figure 4. A). *Tgfbr1* expression varied over time and continued with much higher levels at E13.5, which are detected throughout the whole cerebellar primordium which is mostly localized in the NTZ and Purkinje cell palate (Figure 14. A). In contrast, *Tgfbr2* expression is not detected at any given time points in the sections of the developing mouse cerebellum (Figure 14. B).

Using the Allen DMBA database, we found that *Smad2* expression was strongly high at E11.5 throughout the whole cerebellar primordium, while its expression is almost abolished at E13.5, supporting our Western blotting data (Figure 14. C). *Smad3* ISH data showed its moderate expression in the rhombomere 1 alar plate (r1A) and Purkinje cell plate of the cerebellar primordium at E11.5. Like *Smad2, Smad3* expression is diminished at E13.5 (Figure 14. D).

A


Tgfbr1
E11.5
E13.5
Developing Mouse, E11.5
Specimen Age: E11.5
Symbol: Tgfbr1
Name: transforming growth factor, beta receptor I
Developing Mouse, E13.5
Specimen Age: E13.5
m2F
ICm
isF
isB
isBv
m2A
IC
CbVv
isA
CbV
m2ADLv
CbVm
r1Av
r1A
r1Am
r1Bm
r1B
r1Bv
r2Bm
r2Bv
m2ALv
CbHs
CbH
CbHi
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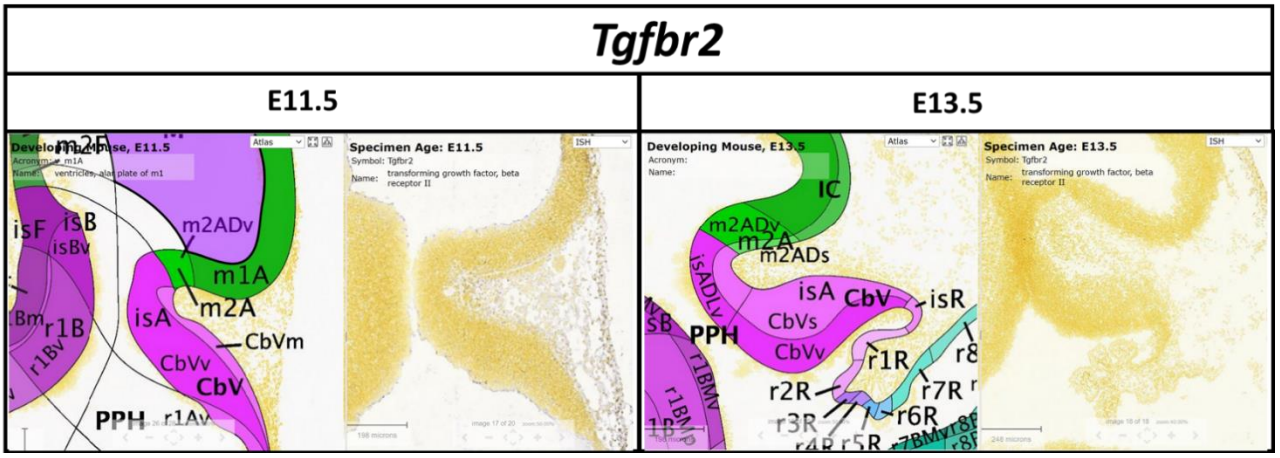
r7R
r1Vev
r1Vel
r1Vep

B


Tgfbr2
E11.5
E13.5
Developing Mouse, E11.5
Specimen Age: E11.5
Symbol: Tgfbr2
Name: transforming growth factor, beta receptor II
Developing Mouse, E13.5
Specimen Age: E13.5
m2F
isF
isB
isBv
m2ADv
m1A
m2A
isA
CbVm
CbVv
CbV
PPH
r1Av
r1B
r1Bv
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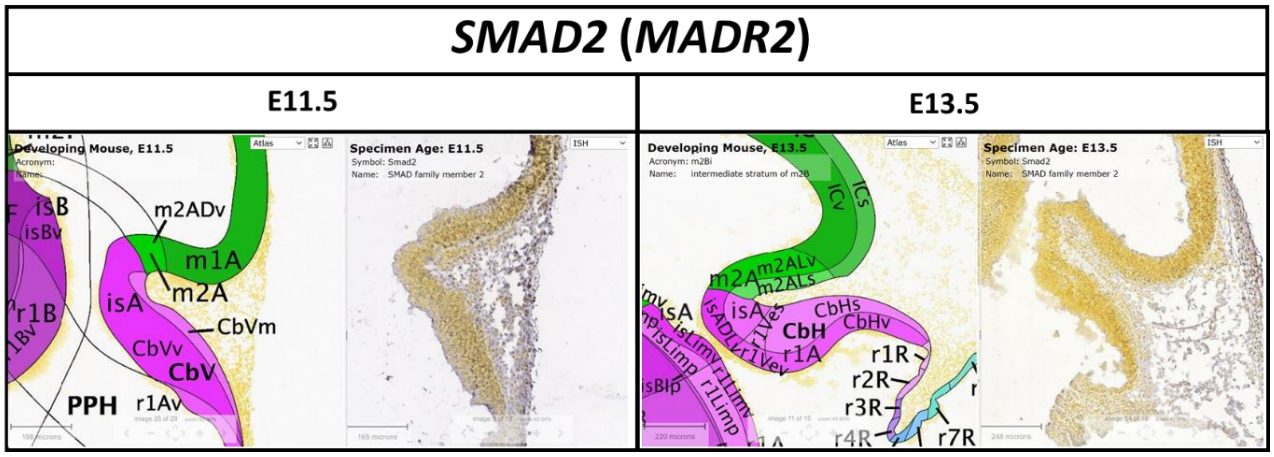
r7R
r6R

C


SMAD2 (MADR2)
E11.5
E13.5
Developing Mouse, E11.5
Specimen Age: E11.5
Symbol: Smad2
Name: SMAD family member 2
Developing Mouse, E13.5
Specimen Age: E13.5
isB
isBv
m2ADv
m1A
m2A
isA
CbVm
CbVv
CbV
PPH
r1Av
r1B
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r2R
r3R
r4R
r7R

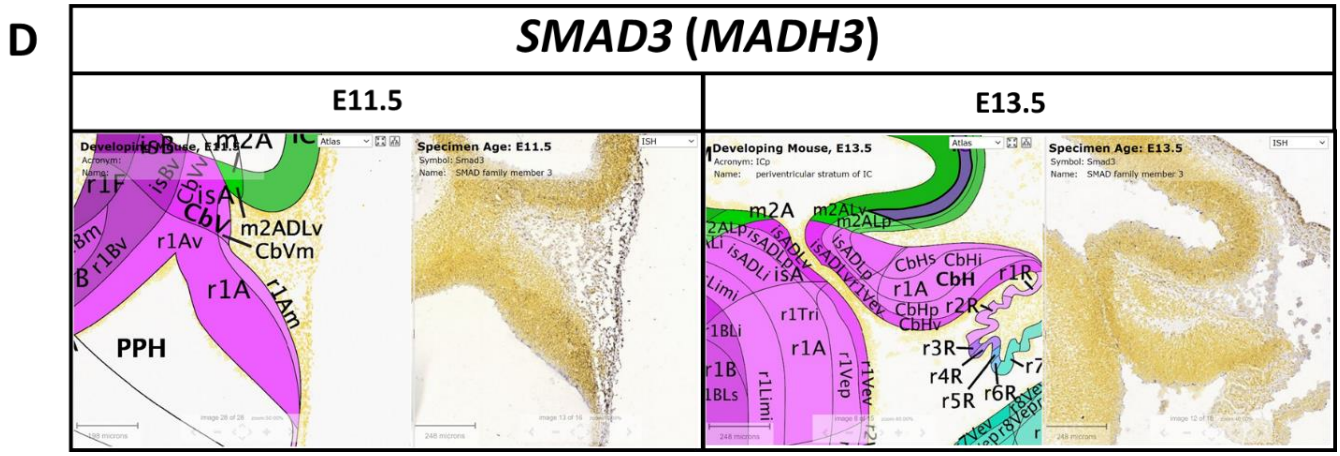

Figure 14. Allen Developing Mouse Brain Atlas ISH data of the Tgfbr1 & 2 and Smad2 & 3 in the developing mouse cerebellum. (A-D) RNA in situ hybridization data from Allen Developing Mouse Brain Atlas was queried for **(A)** Tgfbr1 (http://developingmouse.brain-map.org/experiment/show/100081537), (B) Tgfbr2 (http://developingmouse.brain-map.org/experiment/show/100045666), (C) Smad2 (http://developingmouse.brain-map.org/experiment/show/100046857), and (D) Smad3 (http://developingmouse.brain-map.org/experiment/show/100046667) at E11.5 and E13.5. Selected images of serial sagittal sections at each time point are presented. A matched drawing atlas and ontologies (left image) show the anatomical location of the ISH pattern for each gene at each embryonic day.

3.4 Inverse correlation between TGF-β_1 & Cell Adhesion Molecules expression in cerebellar primordium during early developmental stages at E9-E13.

In our experiments, we confirmed the presence of the canonical TGF-β signaling pathway during the early developmental stages of the cerebellar primordium. In the next step, we wanted to understand the role of the TGF-β signaling pathway and its possible regulatory role through interaction with other signaling pathways. Therefore, we evaluated both mRNA and protein expression levels of a group of most promising cell adhesion molecules such as N-Cadherin/Cdh2, Cdh8, NCAM, and β-Catenin to answer the question if TGF-β_1 regulate the proliferation/migration, and differentiation process. Our results showed an inverse correlation between TGF-β_1 and cell adhesion molecule's pattern of expression in cerebellar primordium across five embryonic days. The downward trend in TGF-β_1 and upward trend in the cell adhesion molecules expression levels. These findings support the idea that high expression levels of TGF-

β_1 during earliest embryonic days at E9 and E10 would activate the canonical TGF-β signaling pathway, transmit the signal to the nucleus through the SMAD family of transcription factors, upregulate the expression of cell adhesion molecules, and finally regulate the neural migration and differentiation process.

3.4.1 Upregulation of N-cadherin/Cdh2 expression in cerebellar primordium at E9-E13.

In general, Cdh2 is a Ca^{2+}-dependent transmembrane glycoprotein that is broadly expressed in neuroepithelial cells during early embryonic and neonatal development. Neural migration and/or differentiation is highly dependent on Cdh2 expression, and variations in its expression change the fate of migration.

In order to evaluate the expression profile of N-cadherin/Cdh2, both protein and mRNA expression levels were measured by Western blotting and RT-qPCR, respectively. Cdh2 protein expression levels showed an upward trend starting from E9 at its lower level to E11, which is significantly higher among all the time points. Consequently, Cdh2 values experienced a downward trend from E12 to E13. Although, the values do not reach statistical significance (Figure 15. A).

Cdh2 mRNA expression values were evaluated by RT-qPCR using RNA samples extracted from mouse embryonic cerebellar tissues. Results showed that *Cdh2* mRNA changing expression by time is not statistically significant between given embryonic days. However, there is an almost constant level of expression with marginal changes from E9 to E13. These data suggest continuous activity of *Cdh2* gene across five embryonic days from E9 to E13. Compared to the Western blot results, in which the pattern in total protein expression is increased from E9 to E11, the unchanged pattern of *Cdh2* mRNA expression suggests the possibility of translation enhancers between embryonic days 9 to 11 in the cerebellar primordium (Figure 15. B).

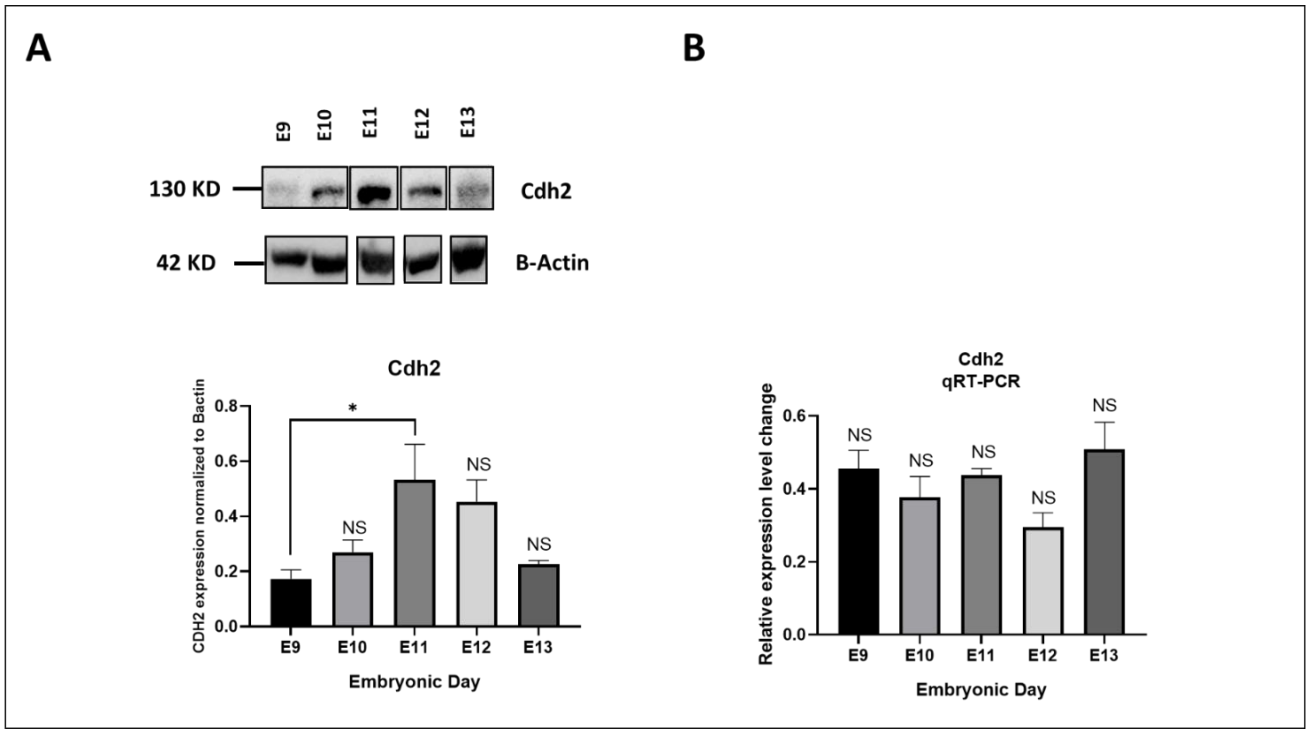

Figure 15. Cdh2 protein expression increased at E11 while its mRNA expression remained unchanged in cerebellar primordium during early developmental stages at E9-E13 (A, B) Expression levels of Cdh2 protein and mRNA in cerebellar tissues derived from CD1 mouse embryos at E9-E13; **(A)** Cdh2 protein expression level was measured by Western blotting. Protein expression is upregulated from E9 to E11 and reached its higher amount at E11, which is statistically significant. The blots were quantified using densitometry software Alpha Ease FC. The protein loading was confirmed using B-actin. The blots are representative of 3 different independent experiments. **(B)** RT-qPCR results showed a constant level of Cdh2 mRNA expression during the earliest embryonic days. This experiment was repeated over three replicates for each embryonic day (n=3). The data in the bar graphs are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA (P-value $\leq$ 0.05 and lower were considered statistically significant).

ISH patterns of *N-Cadherin/Cdh2* on sagittal sections during the cerebellar development support the persistent expression of *Cdh2* over the indicated time points at E11.5 and E13.5. As can be seen, *Cdh2* expression shifts from scattered expression throughout the entire cerebellar section at E11.5 to the condensed pattern in the NTZ and Purkinje cell plate (mostly in Foxp2+ cells) (Marzban, Rahimi-Balaei, and Hawkes 2019) of the cerebellar primordium at E13.5 (Figure 16).

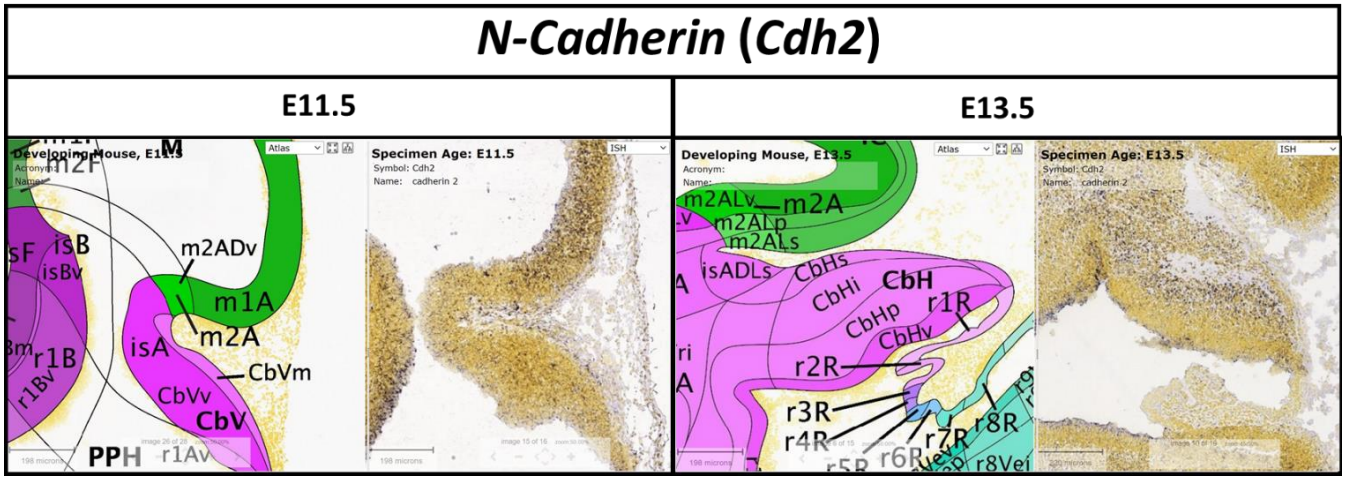

Figure 16. Allen Developing Mouse Brain Atlas ISH data of the N-Cadherin/Cdh2 in the developing mouse cerebellum. RNA in situ hybridization data from Allen Developing Mouse Brain Atlas was queried for Cdh2 (http://developingmouse.brain-map.org/experiment/show/100041183) at E11.5 and E13.5. Selected images of serial sagittal sections at each time point are presented. A matched drawing atlas and ontologies (left image) show the anatomical location of the ISH pattern for the Cdh2 gene at each embryonic day.

3.4.2 Constant expression of β-Catenin in cerebellar primordium at E9-E13.

β-catenin is a multifunctional protein that, under normal physiological conditions, contributes to cellular development by regulating and coordinating cell-cell adhesion. β-catenin is a member of cadherin binding proteins that mediate the intracellular connection of Cadherins (such as Cdh2) and the actin cytoskeleton. In addition, β-catenin regulates the expression of cell adhesion molecules (CAMs) during embryonic development.

Therefore, Western blot was carried out using anti- β-catenin antibody to evaluate the protein expression level of β-catenin in mouse embryonic cerebellar tissue at E9-E13. The expression of the β-catenin protein is maintained at the same level without any significant variation across embryonic days (Figure 17. A).

Extracted RNA samples from embryonic cerebellar tissues were also quantified by RT-qPCR. Results demonstrated that *β-catenin* transcription experienced a downward trend with maximum expression level at E9 and significant reduction at E11 and E12. Subsequently, mRNA values increased moderately at E13; however, the differences are non-significant (Figure 17. B).

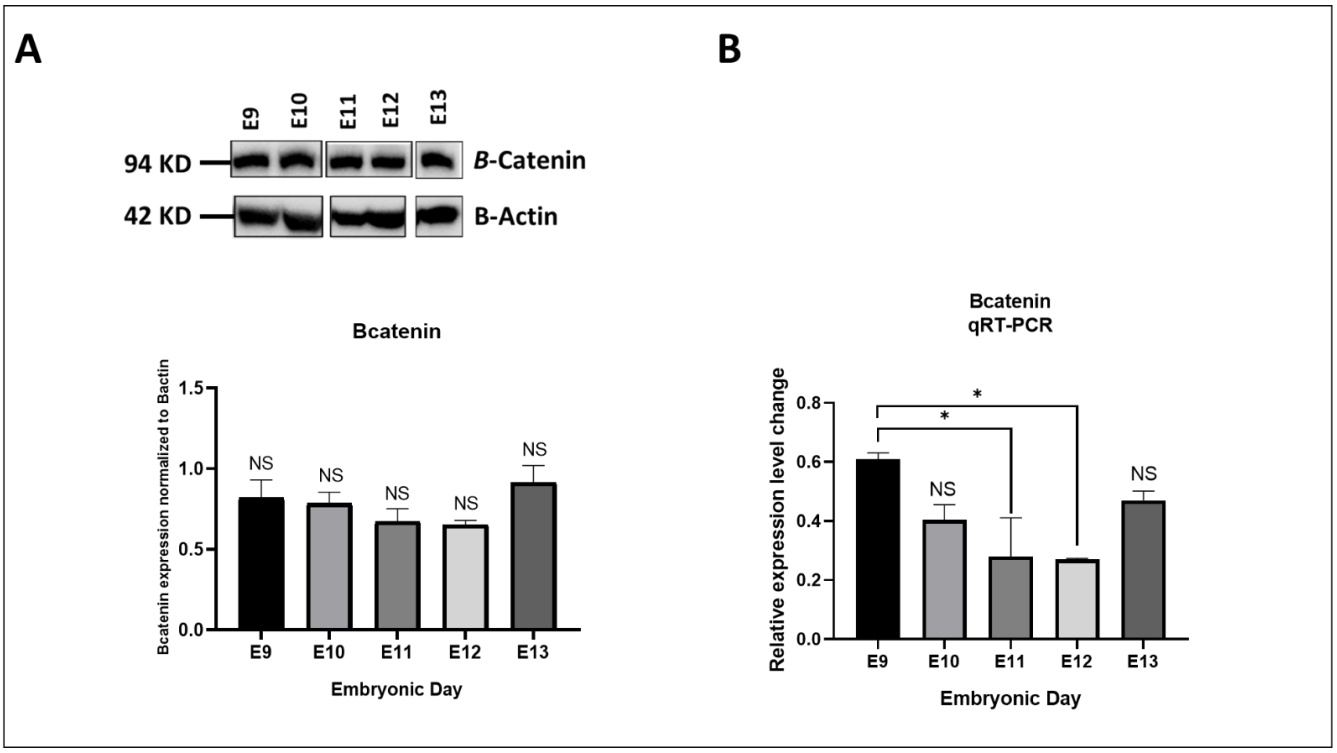

Figure 17. β-catenin protein expression remained unchanged while the trend for mRNA values is negative in cerebellar primordium during early developmental stages at E9-E13. **(A, B)** Expression levels of β-catenin protein and mRNA in cerebellar tissues derived from CD1 mouse embryos at E9-E13; **(A)** β-catenin protein expression level was measured by western blotting. Protein expression remained unchanged with no significant differences between embryonic days. The blots were quantified using densitometry software Alpha Ease FC. The protein loading was confirmed using B-actin. The blots are representative of 3 different independent experiments. **(B)** mRNA expression of β-catenin was measured by RT-qPCR. β-catenin transcription at E9 is higher with significant reduction at E11 and E12. This experiment

was repeated over three replicates for each embryonic day (n=3). The data in the bar graphs are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA (P-value ≤ 0.05 and lower were considered statistically significant).

Allen DMBA showed robust expression of ***β-Catenin*** at both E11.5 and E13.5. Enriched expression can be seen in the entire sagittal sections through the cerebellar primordium at E11.5 and localized in the Purkinje cell plate at E13.5 (less in NTZ). ***β-Catenin*** ISH data confirm Western blot and RT-qPCR results, which indicate constant expression levels of both β-Catenin protein and mRNA expression during the earliest embryonic days from E9 to E13 (Figure 18).

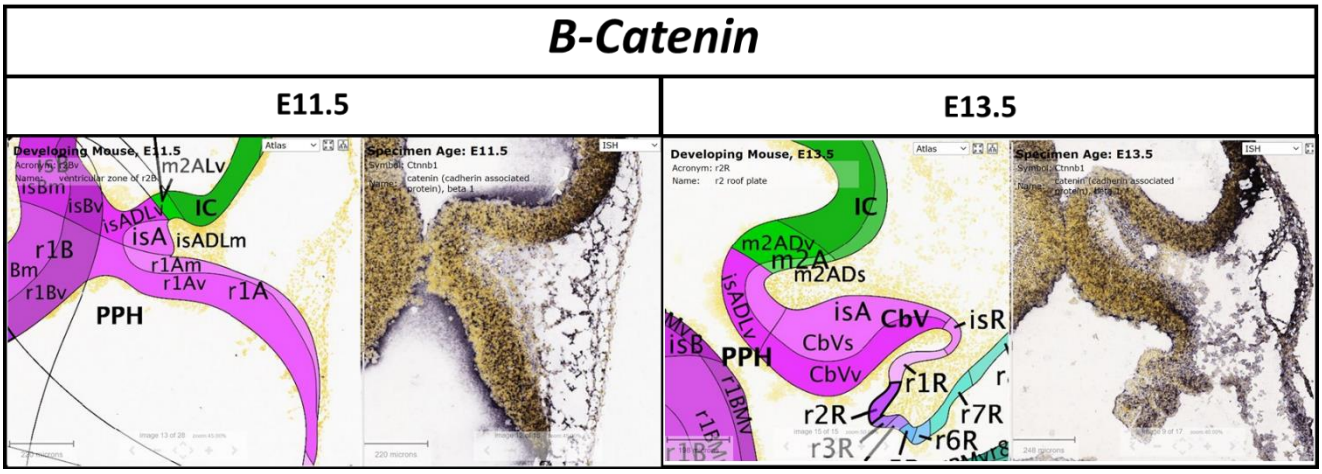

Figure 18. Allen Developing Mouse Brain Atlas ISH data of the β-Catenin in the developing mouse cerebellum. RNA in situ hybridization data from Allen Developing Mouse Brain Atlas was queried for β-Catenin (http://developingmouse.brain-map.org/experiment/show/100077818) at E11.5 and E13.5. Selected images of serial sagittal sections at each time point are presented. A matched drawing atlas and ontologies (left image) show the anatomical location of the ISH pattern for the β-Catenin gene at each embryonic day.

3.4.3 Upregulation of Cdh8 protein and mRNA expression levels in cerebellar primordium at E9-E13.

In our study, Cdh8 is another Ca-dependent cell adhesion molecule expressed in specific domains of the cerebellar cortex including PCs. To examine the changing expression pattern of

Cdh8 in mouse embryonic cerebellar tissues, Western blot was performed using anti-Cdh8 antibody. According to the analyzed data, Cdh8 has no expression during the earliest developmental days from E9-E11. However, Cdh8 protein expression starts at E12 and reaches its highest level at E13, which is statistically significant (Figure 19. A).

Using RT-qPCR, *Cdh8* transcripts are detected at all embryonic days from E9 to E13. In addition, the pattern in *Cdh8* mRNA expression increases gradually from E9 at its lowest level and reaches its highest level at E13, which is statistically significant (Figure 19. B).

As shown in Figure 19., the *Cdh8* mRNA result shared a similar pattern to the total protein trend. Both Cdh8 protein and mRNA values are increased over time with the strong expression and highest values at E13. The positive trend indicates an increasing transcription of *Cdh8* mRNA and consequent expression of Cdh8 protein in cerebellar primordium at E12-E13.

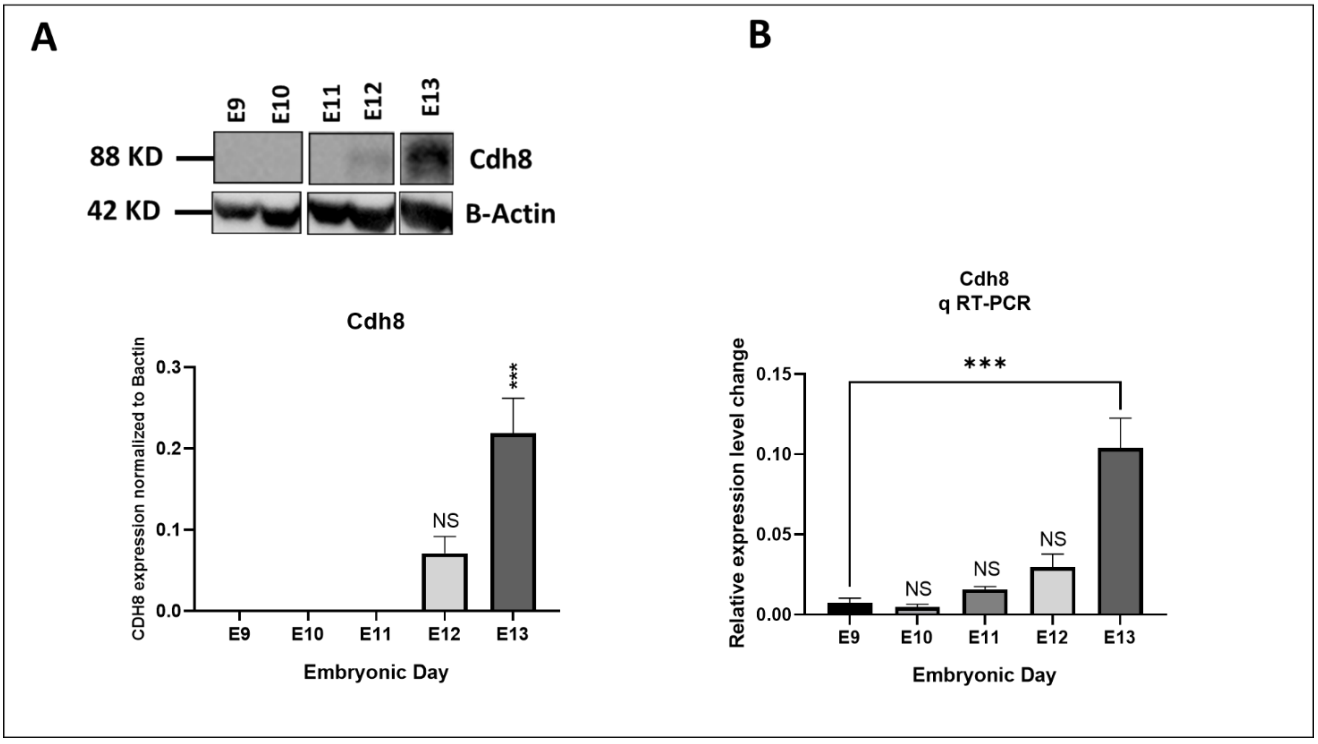

Figure 19. Cdh8 protein and mRNA expression levels experienced an upward trend in cerebellar primordium during early developmental stages at E9-E13. (A, B) Expression levels of Cdh8 protein and mRNA in cerebellar tissues derived from CD1 mouse embryos at E9-E13; **(A)** Cdh8 protein expression level was measured by western blotting. There is no expression during the earliest embryonic days at E9-E11. Protein expression is upregulated from E12 to E13 and

reached its higher amount at E13, which is statistically significant. The blots were quantified using densitometry software Alpha Ease FC. The protein loading was confirmed using B-actin. The blots are representative of 3 different independent experiments. **(B)** RT-qPCR results showed increased levels of Cdh8 mRNA expression from E9 to E13 with lower and higher expression levels at E9/E10, which is significantly increased at E13. This experiment was repeated over three replicates for each embryonic day (n=3). The data in the bar graphs are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA (P-value $\leq$ 0.05 and lower were considered statistically significant).

Using Allen DMBA and RNA ISH data, we found that *Cdh8* is not detected by RNA probes over given developmental timepoints at E11.5, but at E13.5 expression is detectable in Purkinje cell plate (Foxp2 immunopositive Purkinje cells). These results correlate with Western blot, and RT-qPCR analyzed data related to the earliest embryonic days, shown by the lack of Cdh8 protein and mRNA expressions from E9 to E11. However, *Cdh8* ISH data does not show the same protein and mRNA pattern at E13.5 (Figure 20).

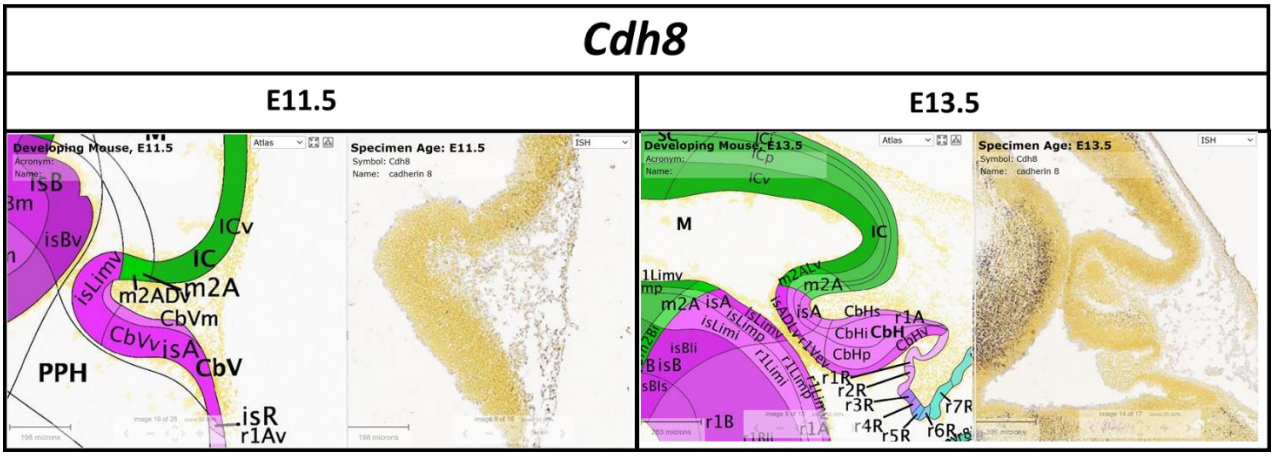

Figure 20. Allen Developing Mouse Brain Atlas ISH data of the Cdh8 in the developing mouse cerebellum. RNA in situ hybridization data from Allen Developing Mouse Brain Atlas was queried for Cdh8 (http://developingmouse.brain-map.org/experiment/show/100077809) at E11.5 and E13.5. Selected images of serial sagittal sections at each time point are presented. A matched drawing atlas and ontologies (left image) show the anatomical location of the ISH pattern for the Cdh8 gene at each embryonic day.

3.4.4 Increasing protein and mRNA expression levels of NCAM in cerebellar primordium at E9-E13.

NCAM is the well-known neural cell adhesion molecule that is expressed on the surface of early embryonic neurons and glia. NCAM plays an essential role in mediating adhesion among neurons and neurite outgrowth. In order to investigate the expression pattern of NCAM protein during early embryonic stages, Western blot was performed using anti- NCAM antibody. According to the analyzed data, there is no expression of NCAM protein during the earliest embryonic days at E9-E11. However, NCAM protein expression sharply increased at E12-E13, which is statistically significant (Figure 21. A). Results from RT-qPCR showed an upward trend in NCAM mRNA expression in cerebellar primordium over five embryonic days. *NCAM* mRNA expression levels increased dramatically from E9 to E13, which shared the same pattern with NCAM protein expression levels (Figure 21. B).

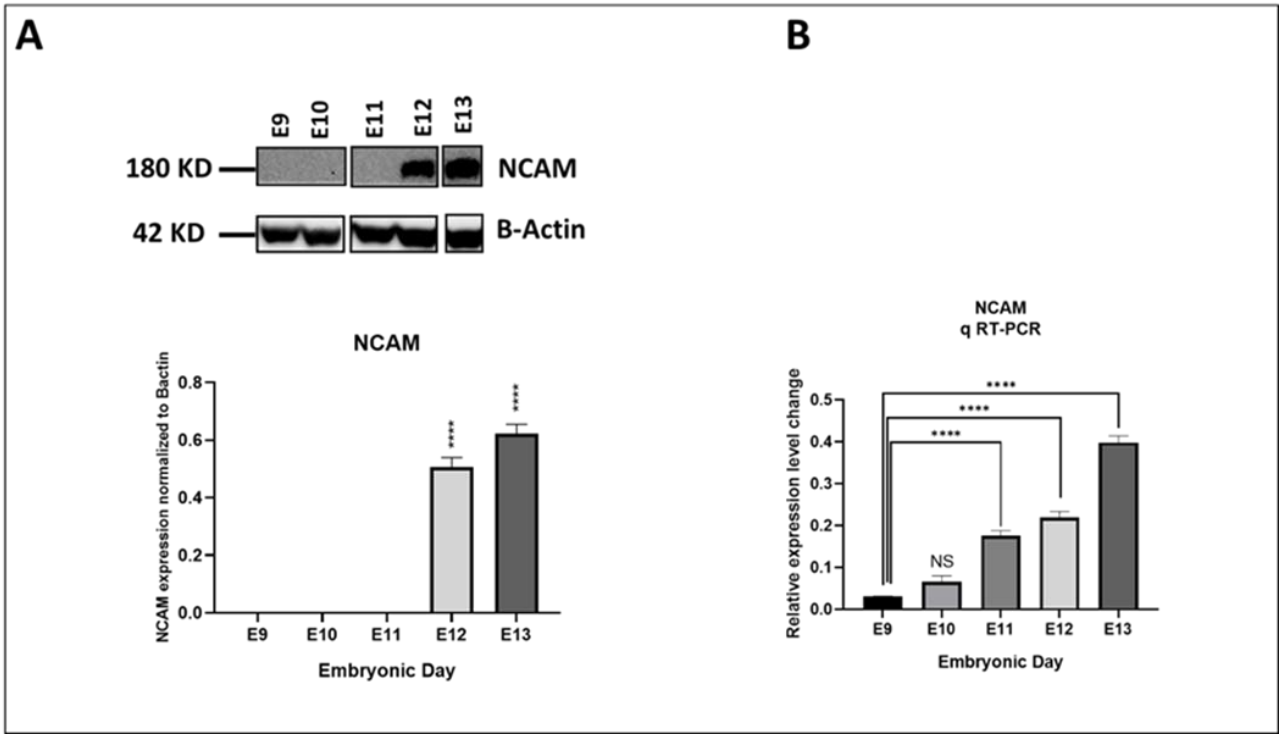

Figure 21. NCAM protein and mRNA expression levels experienced an upward trend in cerebellar primordium during early developmental stages at E9-E13. (A, B) Expression levels of NCAM protein and mRNA in cerebellar tissues derived from CD1 mouse embryos at E9-E13;

(A) NCAM protein expression level was measured by Western blotting. There is no expression during the earliest embryonic days at E9-E11. Protein expression is upregulated from E12 to E13 and reached its higher amount at E13 which is statistically significant. The blots were quantified using densitometry software Alpha Ease FC. The protein loading was confirmed using B-actin. The blots are representative of 3 different independent experiments. **(B)** RT-qPCR results showed increased levels of NCAM mRNA expression from E9 to E13 with lower and higher levels of expression at E9 and E13, respectively. This experiment was repeated over three replicates for each embryonic day (n=3). The data in the bar graphs are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA (P-value ≤ 0.05 and lower were considered statistically significant).

In situ hybridization patterns for *NCAM* showed organized expression pattern at both E11.5 and E13.5. The enrichment in *NCAM* can be seen in the sagittal E11.5 cerebellar section in the NTZ of rhombomere 1 alar plate (r1Am). In addition, Allen DMBA showed strong expression of *NCAM* in the NTZ and Purkinje cell plate at E13.5. These findings confirm our Western blot and RT-qPCR results, demonstrating an increasing amount of protein and mRNA expression over time from E9 to E13 (Figure 22).

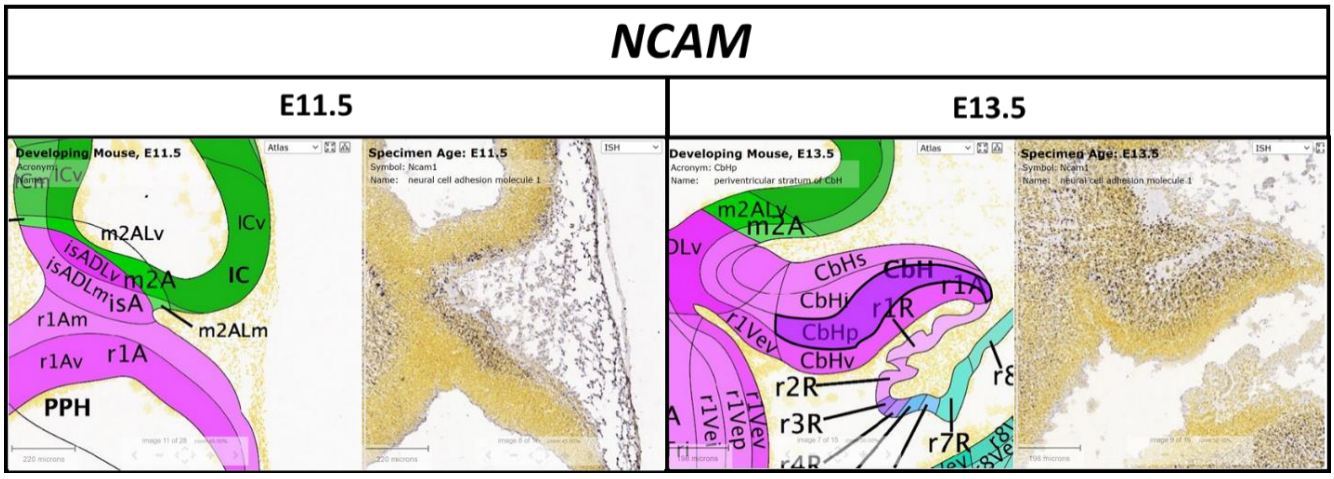

Figure 22. Allen Developing Mouse Brain Atlas ISH data of the NCAM in the developing mouse cerebellum. RNA in situ hybridization data from Allen Developing Mouse Brain Atlas was queried for NCAM (http://developingmouse.brain-map.org/experiment/show/100046871) at E11.5 and E13.5. Selected images of serial sagittal sections at each time point are presented. A matched drawing atlas and ontologies (left image) show the anatomical location of the ISH pattern for the NCAM gene at each embryonic day.

3.5 INHIBITION OF AUTOPHAGY FLUX IN CEREBELLAR PRIMORDIUM DURING EARLY DEVELOPMENTAL STAGES AT E9-E13.

Autophagy is a highly conserved lysosomal degradation and clearance pathway necessary for proper proliferation/migration, differentiation, development, remodeling, and intracellular refreshment (Mcknight, Mizushima, and Yue, n.d.). LC3β immunoblotting is the most widely used method to detect autophagic-flux. Using Western blotting, LC3β is detected as two bands: LC3β-I and LC3β-II at around 16 kDa and 14 kDa, respectively. LC3β-I is a cytosolic protein, and LC3β-II is located on the autophagosome membrane, indicating the number of autophagosomes. Therefore, we used the ratio between LC3β-II and LC3β-I to detect LC3 conversion as the indicator of autophagic-flux, which is defined as a measure of autophagy degradation activity. According to the analyzed data, the LC3β-II/LC3β-I ratio demonstrates an upward trend from E9 to E13, which is statistically significant at E13. This pattern of expression suggests increased degradation activity of autophagy (Figure 23. A).

In the next step, the abundance levels of LC3-βI and II were analyzed separately. Results showed that although there is an upward trend in both LC3β-I & II expression levels, LC3β-II is lower than LC3β-I at each embryonic day (Figure 23. B). Our results also showed increased accumulation of LC3β-II starting E11. As LC3β-II is a marker for lapidated LC3 and formation of autophagosome, it shows that autophagosomes are accumulated from E9 to E13 (Figure 23A)

We further investigate the autophagic-flux by evaluating the degradation of the autophagic adaptor, p62, as a complementary marker to measure the rate of degradation. Western blotting was performed using anti- p62 antibody. As can be seen, p62 protein degradation decreased from E9 to E12, and the differences are statistically significant at E11 and E12. However, the p62 value decrease at E13 and reach its detected level during the earliest embryonic days at E9 and E10 (Figure 23. C). Overall, our results showed the accumulation of autophagosomes due to the decreased degradation rate of these vacuoles and inhibition of the autophagy flux from E9 to E13. These results suggest a potential correlation between autophagy flux and phenotype change during cerebellum development (E9-E13).

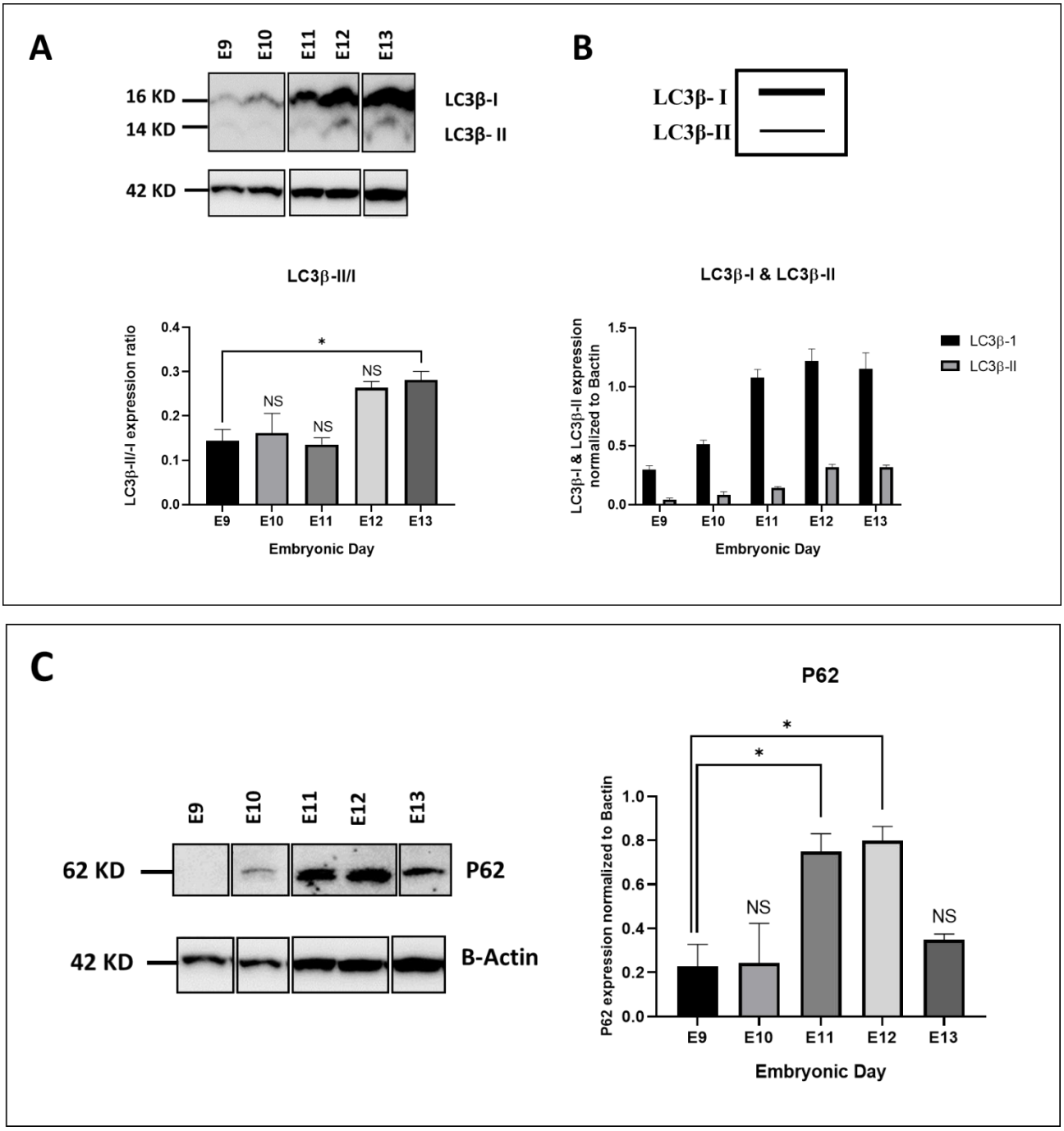

Figure 23. LC3β I & II and p62 protein expression levels experienced an upward trend in cerebellar primordium during early developmental stages at E9-E13. (A, B, C) Expression levels of LC3β-I & II and p62 in cerebellar tissues derived from CD1 mouse embryos at E9-E13; **(A)** LC3β-I & II protein expression levels were measured by western blotting. LC3β-II/LC3β-I ratio increase gradually from E9 to E13, and the values reached the highest level at E13, which is statistically significant. **(B)** Expression levels of both LC3β-I and LC3β-II increase by time; however, LC3β-II value is lower than LC3β-I at each embryonic day. **(C)** P62 protein expression

levels experienced an upward trend from E9 to E12, followed by a rapid reduction at E13. The P62 value is statistically significant at E12. The blots were quantified using densitometry software Alpha Ease FC. The protein loading was confirmed using B-actin. The blots are representative of 3 different independent experiments. Data in the bar graphs are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA (P-value $\leq$ 0.05 and lower were considered statistically significant).

4 CHAPTER IV: DISCUSSION

4.1 OVERVIEW

Mouse cerebellar primordium is established in early embryonic days 7-8 due to the expression of the caudally and rostrally transcription factors and extracellular signals, which the ISO releases (Consalez et al., 2021b; Leto et al., 2016). Coordinated production of the multiple neural cell types within the cerebellar primordium is a critical step during the development of the cerebellum (Fernandes et al., 2012). All cerebellar neurons arise from two distinct germinal epithelial (neuroepithelium): the ventrally located ventricular zone and dorsally-located rhombic lip (Consalez et al., 2021b; Englund, 2006). Generation of all GABAergic neurons starts from E9 in the wall of the VZ of the 4th ventricle, followed by generation, proliferation, and differentiation of the PCs between E10 to E13 and multiple inhibitory classes of inhibitory interneurons after E12. In addition, during this period and before E14.5 (E10-E13), postmitotic and differentiated PCs which complete their final mitotic division at E10.5–12.5 (Rahimi-Balaei et al. 2018), migrate dorsally via the cerebellar plate towards the PCs plate (PCP) to form the distinct clusters of the PCs by E14.5. In parallel, all the glutamatergic neurons including GCPs, and excitatory cerebellar nuclei neuron progenitors, are derived from the URL between E9 to E12 (Fernandes et al., 2012; Rahimi-Balaei et al., 2018; Wang et al., 2005; Wang & Zoghbi, 2001). Projection neurons of the CN have a dual origin, including RL-derived glutamatergic and VZ-derived GABAergic CN neurons, which are born between E9 to E12 with a peak at around E11.5 that migrate to the nuclear transitory zone (NTZ) (Fernandes et al., 2012; Wang et al., 2005). In addition, GCPs migrate over the embryonic cerebellar surface to form the external granular layer (EGL), then proliferate and differentiate for three weeks in this outer layer postnatally. After birth, the postmitotic GCs that leave their axons in the ML and bifurcate to form the parallel fibers, migrate radially through the ML towards the mature granular layer, just superficial to the white matter (Consalez et al. 2021b).

Our study focused on cerebellar tissues collected from mouse embryos during the earliest developmental stages from E9 to E13. Based on the neurogenesis timing of the cerebellum, as mentioned earlier, the only postmitotic neural populations that exist in the cerebellar primordium between E9 to E13 are the PCs and CN neurons located at the PCP and NTZ, respectively. The

principal cytostructure of the cerebellum is established during early developmental stages, followed by neurogenesis and axonogenesis in which cerebellar circuits are formed (Marzban et al. 2015). Therefore, any developmental defect that fails to establish the precise cerebellar circuits will inevitably result in functional and anatomic abnormalities of the cerebellum (Basson & Wingate, 2013; Hibi & Shimizu, 2012). Notably, defects in cerebellar development and components of cerebellar neural circuits have been linked to a range of psychological and pathological disorders, from autism and schizophrenia to ataxia (Hibi et al., 2017; Jimsheleishvili & Dididze, 2019; Keefe & Nowakowski, 2020). Since most of the studies were referring to the experiments conducted based on postnatal development, prenatal studies are very limited in this field. This is another main reason for investigating cerebellar development during the earliest developmental stages from E9 to E13 in our experiment.

The cerebellar development is orchestrated by highly regulated genetic programs and environmental factors (Leto et al. 2016). Multiple studies have shown the crucial roles of genes in cerebellar patterning; however, accumulated information shows that environmental influences play a critical role in the cerebellum and its primordium development. These factors contribute to establishing and maintaining the cerebellar circuit and structure in a spatiotemporal manner (J Xu et al., 2000). Elucidating the regulatory mechanisms behind these extrinsic factors not only increases our understanding of cerebellar development but may also clarify the pathology that suggests possible therapies for cerebellar diseases related to the developmental defects (Hibi et al., 2017; Hibi & Shimizu, 2012). Therefore, for the first time, our lab screened the expression of 57 cytokines, chemokines, and three isoforms of TGF-βs (1-3) in the mouse cerebellar primordium during the earliest developmental stages from E9 to E13. Based on our analyzed results, only 19 cytokines/chemokines and two isoforms of TGF-βs (TGF-β1&2) are expressed in cerebellar primordium during the given developmental period.

We focused our analysis on TGF-β_1 due to its essential roles in CNS development, homeostasis, and repair. Several studies have pointed out the involvement of the TGFβ_1 signaling in a wide range of cellular functions, including EMT and autophagy (Alizadeh et al. 2018). They observed that mice with deficiency in TGFβ_1 experienced increased neural death and microgliosis in the developing cerebellum, which corroborates the critical role of TGFβ_1 signaling in the development of the cerebellum (Alcantara Gomes et al., 2005; Ana P.B. Araujo et al., 2016). In

our study, we realized that the expression of active TGF-β_1 was intensely high at earliest embryonic days at both E9 and E10, followed by a substantial reduction in its value from E11 to E13. These data highlight the possible role of TGF-β_1 during the earliest developmental stages of the cerebellar primordium, which is shown by the overexpression of this cytokine at E9 and E10. In addition, the presence of the activated TGF-β_1 in the cerebellar primordium could be the sign of TGF-β signaling pathway activity during the earliest developmental stages. Additional evidence for the role of TGF-β_1 in cerebellar development is provided by the expression of this growth factor in the cerebellar primordium. Allen DMBA ISH data at E11.5 showed *Tgfb1* RNA expression in the pia mater of the cerebellar primordium, tela choroidea of the fourth ventricle, and a shallow expression level in the neuroepithelium. However, we could not see any expression of TGF-β_1 at E13.5, which is in line with our results.

Therefore, it is hypothesized that TGF-β_1 by upregulating the expression of cell adhesion proteins, including N-cadherin, Cdh8, NCAM, and cadherin binding protein β-catenin, contribute to the physical interactions and connectivity between postmitotic PCs and CN neurons, locating in the PCP and NTZ during earliest developmental stages from E9 to E13 with a peak at E11. In addition, TGF-β_1, by regulating the autophagic-flux, responds to the extracellular signals such as stress (starvation, hypoxia, aggregation of unwanted materials) to maintain cellular homeostasis and provide neural protection during cerebellar development. Our study shows that TGF-β_1 through the canonical TGF-β signaling pathway could upregulate the expression of CAMs. On the other hand, activation of the TGF-β signaling pathway and its consequent reduction in available TGF-β_1 amounts, autophagic-flux, which the TGF-β_1 regularly induces, is inhibited from E9 to E13. **Our findings in this study are consistent with each other and highlight the possible regulatory role of TGF- β_1 in the expression of cadherins and autophagy flux during the earliest stages of cerebellar development.**

4.2 Activation of the Smad-dependent TGF-B_1 signaling pathway in the mouse cerebellar primordium at E9-E13.

Further, we discuss whether and how TGF-β_1 might contribute to the early development of the cerebellum. Our finding showed the presence of main components of the canonical TGF-β

signaling pathway, including TGFβRI, TGFβRII, and cytoplasmic total and phosphorylated SMAD signaling molecules during the given embryonic period. Based on our immunoblot results, upregulation of both activated TGF-β_1 and cytoplasmic total SMADs signaling molecules at E9 and E10, and presence of both *Tgfbr1* and *Tgfbr2* mRNA suggest the participation of the canonical TGF-β signaling pathway during early developmental stages of the cerebellar primordium. Based on the data from TGF-β 3-plex array, TGF-β_1 changed substantially with time and showed a downward trend from E9 to E13 with the highest levels of expression during the earliest developmental stages at E9 and E10, followed by dramatic reduction from E11 to E13. Similarly, total SMAD 2 and 3 protein levels experienced a downward trend from E9-E13 with higher expression levels at E9 and E10. It has been reported that mouse model with mutations in downstream effectors of the TGF-β_1 signaling pathway, including SMAD2, 3, and 4, experienced cellular and structural deficits in developing cerebella such as reduction in dendritic arborization of the PCs and smaller size of the cerebellum (Ana P.B. Araujo et al. 2016).

Our data in this study correlates with Allen DMBA ISH data, where they showed that total *Smad2* RNA expression is intensely high in the entire cerebellar anlage, whereas *Smad3* expressed moderately in the rhombomere 1 alar plate (r1A) and PCP. However, RNA expression of both factors is reduced at E13.5.

This reduction in total SMAD 2 & 3 protein levels can be described as the activation of the TGF-β signaling pathway upon binding of the TGF-β1 ligand to the heterotetrameric complex of the receptors, and consequent conversion of total SMAD 2 & 3 to phosphorylated and activated SMADs. We have also shown that protein expression levels of phosphorylated SMAD 2 had no signals during the earliest embryonic days at E9 and E10, and its signals started to appear from E11 with higher amounts at E12 and E13. Based on the abundance and availability of TGF-β1 ligands, the duration and intensity of the Smad-dependent transmitted signals are regulated (Hata and Chen 2016). It is believed that both glutamatergic CN neurons and GABAergic PCs are born with a peak at around E11.5 (Rahimi-Balaei et al. 2018). Based on our findings, at this stage (E11.5) TGF-β signaling pathway has already started, which is shown by depletion of the TGF-β1 ligands, reduction of total SMAD 2 and 3 protein levels, and elevated levels of phosphorylated SMAD 2. **Therefore, these promising findings highlight the possible activity and role of the**

Smad-dependent TGF-β signaling pathway in the formation of the PCs clusters and localization of the CN neurons in the cerebellar primordium from E9 to E13.

The other important factor that regulates the duration and intensity of the TGF-β_1 signaling pathway is the expression level and cell-surface distribution of the type I and type II TGF-β receptors (Hata and Chen 2016). However, the cerebellar expression of TGFβRI and GFβRII during embryonic developmental stages are unknown, especially at earliest embryonic days from E9 to E13. In our study, RT-qPCR results demonstrated that both *Tgfbr1* and *Tgfbr2* mRNA express across the given embryonic period. Interestingly, *Tgfbr1* mRNA expression level had a downward trend from E9 to E13, with higher and lower amounts at E9 and E12, respectively. However, there is an almost constant level of *Tgfbr2* mRNA expression from E9 to E13. One possible reason is that ligand depletion in the TGF-β network occurs through TGFβRII (Zi et al., 2012). Therefore, the constant expression level of *Tgfbr2* shows continuous depletion of the TGF-β_1 ligands, which contributes to the amplification and duration of the Smad-dependent signaling. It has been reported that cells with defects in expression of TGFβRII, but not TGFβRI, were unable to deplete TGF-β_1 ligands from the environment (Zi et al., 2012). In our study, Allen DMBA ISH data demonstrated that *Tgfbr2* mRNA expression is not detected at selected embryonic time points.

One possible explanation to account for this observation could be the depletion of TGF-β_1 ligands through TGFβRII-mediated endocytosis. It should be noted that the activated ligand-receptor complex is internalized via two main types of endocytosis, including clathrinid-dependent and clathrin-independent processes that serve as a mechanism for down-regulation of TGF-β signaling. Internalized and activated ligand-receptor complexes are delivered into early endosomes containing SARA proteins, contributing to the phosphorylation of SMAD2 and 3 and their connection with SMAD4 (Co-SMAD) (Zi et al., 2012). Therefore, internalizing the TGFβRII accompanied by depletion and removing the active TGF-β_1 ligands from the cell surface could act as a primary termination signal that represses *Tgfbr2* gene transcription and reduces the production of the *Tgfbr2* mRNA, which all lead to the inactivation of TGF-β signaling.

The other possibility could be the neurotrophic action of the TGF-β1 through Brain-Derived Neurotrophic Factor (BDNF). Neurotrophic factors are endogenous substances that control the proliferation and differentiation of the neural cells through their specific receptor (Lu et al. 2005). Based on the studies, TGF-β1 enhances the expression of BDNF and its receptor TrkB in terms of

both mRNA and protein in neurons cultured from rat cerebral cortex (Sometani et al. 2001). The BDNF as a ligand and Trkb as a neurotrophin receptor activate multiple signaling pathways that provide crosstalk to decide if the cell needs to survive, proliferate, or differentiate. Therefore, TGF-β1 could indirectly and through increased BDNF and TrkB protein and mRNA expression regulate critical cellular events during early cerebellar development (Lu et al., 2005; Sometani et al., 2001).

Moreover, Various developmental events are dependent on cell-cell/cell-ECM interactions, such as cell-cell adhesion and cell migration (J. Luo 2005). It has been reported that TGF-βs, by facilitating the accumulation of the extracellular matrix (ECM) components, regulate cell growth and differentiation (Sometani et al. 2001). The matrix metalloproteinase (MMP) family are proteolytic endopeptidases that, by degrading the protein components, participate in the remodeling of the ECM (Wee Yong et al. 2001). Some members of metalloproteinases are expressed in the developing CNS, indicating their possible relevance to neural development (J. Luo 2005). The tissue inhibitors of metalloproteinases (TIMPs) negatively regulate the activity of the MMPs, which is a potent contributor to ECM remodeling (Ries, 2014). Therefore, the balance between MMPs and TIMPs and their constant activity is necessary for mediating diverse neurodevelopmental processes which are orchestrated by the cell-ECM interactions (Hall et al., 2003; J. Luo, 2005). Also, TIMPs regulate additional biological events such as cell growth, differentiation, and apoptosis by their cytokine-like activities (Ries 2014). It has been reported that TGF-β1 through Smad-dependent and Smad-independent MAPK signaling pathways alter the levels of *Timp-1* and *MMP-1* at the gene expression level, which leads to the ECM remodeling and homeostasis. Consequently, *MMP-1* gene transcription is suppressed, and *Timp-1* gene expression is induced (Hall et al. 2003).

Based on our results, TIMP-1 expression has the highest rate among all the examined cytokines. Its expression experienced dramatic growth from E9 to E10 and reached its highest level at E10 which is coincide with the highest levels of active TGF-β1 at E9/E10. Consequently, TIMP-1 expression amounts showed a downturn and hit the lowest point at E13. Therefore, based on our data activated TGF-β1 through the Smad-dependent signaling pathway could induce and upregulate the expression of TIMP-1, which is associated with the remodeling and homeostasis of the ECM and the regulation of various neurodevelopmental signaling pathways in the mouse cerebellar primordium at E9-E13.

4.3 TGF-β_1 upregulate the expression of Cadherins in the mouse cerebellar primordium at E9-E13.

Our study shows that TGF-β_1 could upregulate the expression of a group of cell adhesion molecules (CAMs) during the earliest developmental stages of the cerebellar primordium. Several studies highlighted the importance of cadherin functions in orchestrating cellular shape, integrity, gathering, and scattering in distinct germ layers during morphogenesis (Hiraga et al. 2020). All these processes require intimate cell-cell contacts and communications coordinated by cadherins (Paulson et al. 2014). In addition, cell adhesion molecules mediated the formation of the functional neural circuits, which occur due to the proper and stable connections between neural processes (Tan et al. 2010). Based on the current studies, cadherins expression during CNS development regulate at different translational and post-translational levels. In addition, cadherin genes are regulated in an appropriate spatiotemporal manner by the signaling pathways that are activated in those regions (Paulson et al. 2014). It has also been reported that during early developmental stages, the expression of cell adhesion molecules is directly or indirectly regulated by multiple overlapping transcription factors. Therefore, alteration in expression of regulatory transcription factors leads to perturbation of cadherin protein expression and consequent abnormal embryonic development (Rogers et al., 2018). Here, we show the possible regulatory mechanism of cadherins at the transcriptional level during cerebellar development, which is controlled by the activated TGF-β signaling pathway. In our study, we evaluate the expression patterns of type I and type II classical cadherins, including N-cadherin (Cdh2) and Cdh8, respectively. In addition, the expression profile of cadherin binding protein β-Catenin and NCAM are also determined.

In vitro and *in vivo* studies demonstrated that increased expression of surface N-cadherin/Cdh2 is necessary for the gathering and maintenance of naïve dendrite arbors (Tan et al. 2010). Based on our western blot results, we have found that protein levels of Cdh2, as a type I classical cadherin, experienced an upregulation from E9 to E11 followed by a reduction in its amounts from E12 to E13. In line with our previous results, which demonstrate the activation of the TGF-β signaling pathway during the peak of PCs and CN neurons generation at E11.5, N-cadherin expression values reached its highest levels at E11, which is statistically significant. Generally, Cdh2 participates in strong homophilic cell-cell adhesion, which leads to the structural integrity of the cortical structures (Paulson et al. 2014). Homophilic interactions require the

expression of the same cadherins by cells that are in physical contact. Therefore, N-cadherin is not able to interact with type II classical cadherins such as Cdh8 (Rogers et al., 2018). However, type I N-cadherin is reported to mediate weak heterophilic connections with cells expressing R-cadherin and E-cadherin (Paulson et al. 2014). Homophilic interactions by cadherins are an essential feature that contributes to cell sorting and tissue integrity (Rogers et al., 2018). Some studies provide evidence that proliferating neural progenitors located in germinal zones of the developing and adult brain are connected through interactions mediating by N-cadherin, which is expressed by these cells (Paulson et al. 2014). Interestingly, data from Allen DMBA ISH reference showed the expression of *Cdh2* at both E11.5 and E13.5, which upregulates and becomes more robust in both PCP and NTZ at E13.5. This expression pattern justifies the role of Cdh2 in both postmitotic cell populations within the cerebellar primordium over time as they develop more intercellular connections. In this regard, our further analysis showed that there is an almost constant level of expression with marginal changes in *Cdh2* mRNA expression from E9 to E13. These data suggest continuous activity of the *Cdh2* gene across five embryonic days from E9 to E13. Compared to the western blot results, in which the pattern in total protein expression is increased from E9 to E11, the unchanged pattern of *Cdh2* mRNA expression suggests the possibility of translation enhancers between embryonic days 9 to 11 that correlates with the generation period of PCs and CN neurons in the cerebellar primordium. **In our scenario, upregulated N-cadherin/Cdh2 by activated TGF-β signaling pathway could maintain postmitotic PCs and CN neurons and form the NTZ and PCs clusters in PCP.**

Many studies have demonstrated that the major intracellular components that link the extracellular ectodomains of the cadherins to the actin cytoskeleton are the catenin proteins (Kwiatkowski et al., 2007; Rogers et al., 2018; Tan et al., 2010). Type I cadherins such as N-cadherin, together with β-catenin and αN-catenin form the cadherin/catenin complex, which is essential for normal cadherin functions, including cadherin-mediated cell adhesion that regulates multiple signaling pathways (Rogers et al., 2018; Tan et al., 2010). It has been reported that high levels of cadherin/catenin complexes are present in synaptic junctions that are formed on both axons and dendrites of the neurons (Kwiatkowski et al., 2007; Tan et al., 2010). In this regard, we further analyzed the protein and mRNA expression levels of β-catenin in cerebellar primordium. Our results demonstrate that although protein levels of β-catenin remained almost unchanged from E9 to E13, *β*-

catenin mRNA values significantly decreased over time, with the highest and lowest amounts at E9 and E11/E12, respectively.

In contrast to the previous studies, our data show that elevated levels of Cdh2 do not affect levels of *β-catenin* mRNA expression from E9 to E13. We believe that the downward trend in *β-catenin* mRNA expression levels from E9 to E12 suggests the idea that β-catenin may not be a critical mediator of Cdh2 expression in postmitotic neural populations over their generation period. However, ISH data in Allen DMBA show extensive and constant expression of *β-catenin* throughout the whole cerebellar primordium at both E11.5 and E13.5, like *the Cdh2* expression pattern. Interestingly, the β-catenin expression pattern over time and at E13.5 become more condensed in the PCP, and its expression attenuates in the NTZ. Therefore, when Cdh2 is expressed, the expression of β-catenin is necessary for membrane localization of the Cdh2 in NTZ and PCP. Collectively, these findings show that constant protein expression of β-catenin would ensure the expression and upregulation of *Cdh2* from E9 to E11. Accordingly, *β-catenin* promotes the cell-cell adhesion function of the Cdh2, leading to the gathering and integrity of the CN neurons and PCs in NTZ and PCP in the cerebellar primordium over five embryonic days E9 to E13. **These data show the direct association between upregulation of both N-cadherin/Cdh2 and β-catenin upon activation of the TGF-β signaling pathway, correlated to time points during which PCs and CN neurons are born and gathered in cerebellar primordium.**

To further determine the expression profile of type II cadherins in earlier developmental stages of the cerebellar primordium, we evaluated the protein and mRNA expression levels of Cdh8. In our study, Cdh8 is a member of type II classical cadherins that mediate less robust heterophilic cell-cell adhesions (Paulson et al. 2014). Type II cadherin expression analyses reveal lack of Cdh8 protein expression from E9 to E11. Cdh8 protein commences to express at E12 and reaches its highest values at E13. However, *Cdh8* mRNA expresses at all embryonic days, which are relatively week before E13. In contrast to type I classical cadherins that are broadly expressed in the central nervous system, type II cadherins are mainly expressed in specific neural circuits and subcellular compartments of the brain (Frei et al. 2020). It has been reported that during development and synapse formation, Cdh8 demarcates the entire cortex, hippocampus, and thalamus (Hiraga et al. 2020). In addition, Cdh8 is an essential adhesion molecule for maintaining neural networks that can support connections between PCs in the clusters (Korematsu et al. 1998).

In line with our western blot results, ISH data from Allen DMBA demonstrated that RNA probes could not detect *Cdh8* mRNA at E11.5. In addition, *Cdh8* mRNA was not detectable at E13.5. One possible reason for this pattern of expression could be the differential expression patterns between type I and type II cadherins (Frei et al. 2020). The temporal expression analysis showed that Cdh2 as a member of the type I classical cadherins expressed with an upward trend from E9 to E11, followed by a reduction in its amounts from E12 to E13. In contrast and in a compensatory manner, type II cadherin-Cdh8 expression starts at E12, increasing significantly at E13. Therefore, a shift in the expression between Cdh2 and Cdh8 may regulate developmental events in cerebellar primordium from E9 to E13. This shift exhibits peak expression of Cdh2 and lack of Cdh8 expression at the time window that coincides with the activation of the TGF-β signaling pathway at around E11/E12.

Moreover, the different temporal expression patterns of Cdh2 and Cdh8 could indicate their unique regulatory mechanisms across the cerebellum. However, the lack of *Cdh8* mRNA expression at both E11.5 and E13.5 suggests that Cdh8 may not be an essential adhesive molecule for gathering both CN neurons and PCs before E12 and imposed its effect at later stages of clusters formation (E12 /E13) by providing weak heterophilic cell-cell adhesions. **Therefore, the activated TGF-β signaling pathway may upregulate the expression of type I and II cadherins, N-cadherin/Cdh2 and Cdh8, sequentially and temporally, associated with proliferation, migration, and positioning of the PCs and CN neurons in PCP and NTZ, respectively.**

The neural cell adhesion molecule (NCAM) is a transmembrane glycoprotein that primarily acts as ligands in the formation of cell-cell contacts (Bahr et al., 1993; Dan Goldowitz et al., 1990; Roubin et al., 2000). Studies showed participation of the NCAM in important events during the development of the CNS, including axonal outgrowth and regeneration, cell migration, and cell-cell interactions of neural and non-neural cells (Bahr et al., 1993; Dan Goldowitz et al., 1990). During embryogenesis, NCAM expression is precisely controlled in all three germ layers derivatives, such as the notochord and the neural crest, to regulate cellular patterning, differentiation, and integrity (Roubin et al., 2000). Our study shows overlapping expression traits of Cdh8 and NCAM in the mouse developing cerebellum from E9 to E13. Like Cdh8, NCAM proteins exhibit a dramatic increase at E12 and E13, indicating their low effects at the earlier stages

of the cerebellar development, from E9 to E11. This surge in expression strongly indicates a specific role for NCAM in regulating cerebellar development at E12 and E13. It has been reported that higher levels of NCMA protein expression were coincident with the terminal stages of PCs dendritic growth into the ML in the developing cerebellum at postnatal day 3 (P3). However, lighter immunopositive staining was associated with the external granular layer in the developing cerebellum (Dan Goldowitz et al. 1990). In our study, overlapped protein and mRNA expression of NCAM and Cdh8 support the idea that Cdh8 and NCAM, which are expressed simultaneously, are involved in regulating the formation of the PCs clusters and NTZ during later stages at E12 and E13. In addition, the expression pattern of *NCAM* using in situ hybridization for mRNA at the level of the cerebellum shows that *NCAM* is enriched in similar cerebellar regions, including NTZ and PCP, that N-cadherin is expressed at both E11.5 and E13.5. Therefore, our data from NCAM may suggest that NCAM has a spatial and temporal correlation with N-cadherin and Cdh8 expression, respectively. Roubin et al., demonstrated that among various growth and differentiation factors, exogenous transforming growth factor-beta stimulated NCAM protein and mRNA expression in 3T3 cells and in early-passage embryo-derived cultures, which highlighted the involvement of TGF-β in the regulation of NCAM expression during embryogenesis (Roubin et al., 2000). **In agreement with this study, activation of the TGF-β signaling pathway at E11 in embryonic NTZ and PCP areas has led to the suggestion that TGF-β may participate in the connection and integrity of the CN neurons and PCs by promoting the expression of NCAM at E12 and E13.**

4.4 TGF-B_1 INHIBIT AUTOPHAGIC-FLUX IN THE MOUSE CEREBELLAR PRIMORDIUM AT E11-E12.

Our study shows that the canonical TGF-β1 signaling pathway potentially correlated with autophagy flux inhibition in cerebellar primordium during the earliest embryonic stages. Based on our immunoblot results, we have found that LC3β lipidation and its conversion to LC3β-II is increased from E9 to E13, which is statistically significant at E13. In general, this pattern of expression and lipidation of LC3β-II suggests the increased formation of autophagosomes or decreased degradation from E9 to E13. On the other hand, p62 degradation was decreased (E9-E13); therefore, we concluded that accumulation of the autophagosomes was due to the decrease in the rate of their degradation and potential inhibition of the autophagy flux (E9-E13).

A body of evidence has increasingly shown that the number of autophagosomes is infrequent in healthy neurons and under normal conditions. One explanation for this scarcity could be the presence of an efficient basal autophagic flux in neurons (Benito-Cuesta et al. 2017). Generally, autophagy act as a protective process in neural cells to maintain the homeostatic balance (Damme et al., 2015; Mcknight et al., 2012). In addition, induction of autophagy in response to different stresses such as starvation and hypoxia in postmitotic neurons, which cannot be regenerated, lead to loss of mass and cells. Therefore, the formation of autophagosomes is tightly controlled in neurons and occurs in a constant low synthesis rate with an extremely high rate of degradation, which helps to turnover of the macromolecules and energy in neurons (Mcknight et al., 2012; Nikolopoulou et al., 2017). Hence, in the normal brain, basal levels of autophagy continuously happen at very low levels due to the efficient autophagy-lysosomal degradation in neurons (Mcknight et al., 2012; Nikolopoulou et al., 2017).

Our data in this study correlates with studies as mentioned earlier where we showed that during the earliest developmental stages at E9 and E10, autophagy flux inhibition is not potent, which is probably involved in providing sufficient nutrients such as amino acids for protein synthesis and eliminating unwanted/accumulated cytoplasmic components within the neural cells. Subsequently, very high and significant levels of p62 are detected at E11 and E12, which indicates accumulation of autophagosomes and inhibition of the autophagy flux in the cerebellar primordium. On the other hand, and in line with our previous results, the formation of the PCs clusters and localization of the CN neurons in NTZ occurs with a peak at around E11.5, which correlates with activation of the Smad-dependent TGF-β signaling pathway at E11 and E12 in the cerebellar primordium. Therefore, the activated TGF-β signaling pathway inhibits autophagy to support neural survival by preventing them from undergoing extensive autophagy and its consequent loss of mass and cells, suggesting that a maximum number of postmitotic PCs and CN neurons can aggregate to form distinct domains within the cerebellar primordium. At E13, P62 levels decrease, and autophagic-flux starts to go back to its normal and basal levels, sufficient for healthy neural cells. **The results from our study pinpoint a potential regulatory role of autophagy inhibition by Smad-dependent TGF-β signaling pathway in the formation of the PCs clusters and accumulation of CN neurons in NTZ during cerebellar development at E11 and E12.**

5 CHAPTER V: CONCLUSION

During the early stages of the mouse cerebellar development, two postmitotic and differentiated neural populations, including PCs and CN neurons, form distinct domains within the cerebellar primordium. In the present study, using mouse embryonic cerebellar tissues derived from E9 to E13, we first showed the activation of the Canonical TGF-β signaling pathway at the time window that coincides with the formation of the NTZ and PCP. Then, we revealed its regulatory effects on Cadherins expression and autophagic-flux during cerebellar development. TGF-β1 may do this in part by promoting the expression of cadherins and inhibition of autophagic-flux. Activated TGF-β signaling pathway sequentially and temporally could upregulate N-cadherin/Cdh2 and β-catenin during earliest stages with maximum expression at E11/E12, and subsequently, start to upregulate the expression of Cdh8 and NCAM at E12 and E13. Therefore, type I and type II cadherins could complement each other's effect by their sequential expression pattern. TGF-β-induced N-cadherin together with β-catenin contributes to the connection and integrity of the neural cells by forming the robust cell-cell adhesions between CN neurons and PCs in their domains, from E9 to E11. Afterward, at E12 and E13, Cdh8 and NCAM, with their overlapping overexpression pattern, continue to support the neural network by forming weaker connections. Our data also showed that basal autophagy acts as a protective process in neurons (neuroprotection) to maintain neural homeostasis during the earliest developmental stages from E9 to E10. However, the activated TGF-β signaling pathway, at E11 and E12, inhibits autophagy to support neural survival by preventing them from undergoing extensive autophagy and its consequent loss of mass and cells. Therefore, our results altogether suggest that the Canonical TGF-β signaling pathway may contribute to the gathering and connection of a maximum number of postmitotic PCs and CN neurons by upregulation of cadherins expression and inhibition of autophagic-flux at E11 and E12. In conclusion, defining the regulatory processes that occur in embryonic development will be fundamental in understanding the mechanisms involved in neurodevelopmental disorders such as autism spectrum disorders and attention-deficit hyperactivity disorders, in which neuronal morphology and structural connectivity are affected during development.

6 CHAPTER VI: LIMITATIONS & FUTURE DIRECTIONS

This study is the first comprehensive developmental based attempt to evaluate the expression of the TGF-β signaling pathway components and its regulatory effects on cadherins expression and autophagy in cerebellar primordium during earliest embryonic days from E9 to E13. Although all effort was put into optimizing the study, there were some potential limitations. Due to the tiny size of the embryo and particularly cerebellar anlage during the earliest embryonic days (E9/E10), there was a small percentage of error when dissecting and removing the cerebellum from the embryo. In addition, the E9 embryo was the smallest size that we were able to start our study. Therefore, we cannot cover the events that occur at the time when cerebellar primordium is established (E7-E8.5) and provide a definitive answer for underlying mechanisms that occur before E9. Due to the time limitation, the sole purpose of using Allen DMBA in our study was to show the mRNA expression of downstream components of the TGF-β signaling pathway and Cadherins. However, a high or low level of mRNA expression does not necessarily warrant the same pattern at the protein level. In addition, ISH data cannot provide fine specificity between different cell types; however, it provides anatomic and morphological data. Therefore, it would be necessary to visualize the protein expression of all the markers by IHC and detect their colocalization in the target cells by IF. In addition, Allen DMBA only provides ISH data at E11.5 and E13.5, so it does not cover the earliest embryonic days at E9/E10.

For the future direction of this study, some more experiments need to be done to address some important questions. First, it is necessary to evaluate the activation and participation of non-canonical TGF-β signaling pathways, including MAP kinase, Rho-like GTPase, and PI3K/Akt during cerebellar development.

Second, it needs to investigate the underlying mechanism in which TGF-β upregulates the expression of the cadherins. In other words, it should be clarified that whether TGF-β directly and through the SMAD family of transcription factors upregulate the expression of cadherin genes or TGF-β by mimicking the process in which EMT is induced reprogram cadherin genes expression. EMT process is characterized by a switch in cadherins expression, from E- to N-cadherin (Thiery et al. 2009). This shift in expression is mediated through families of transcription factors such

as *Sox, Snail, Slug,* and fork head box D3 (*FoxD3*). The expression of these transcription factors is induced by the TGF-β signaling pathway (Kalluri & Weinberg, 2009; Jian Xu et al., 2009). Upon activation of the TGF-β signaling pathway, these transcription factors, in turn, reprogram cadherin genes expression (Jian Xu et al., 2009). Therefore, by evaluating the expression of these families of transcription factors, we can elucidate the underlying mechanism in which cadherins expression is upregulated.

Third, it is better to confirm the results of the autophagy flux and its role during the formation of the PCP and NTZ by co-labeling the LC3β and P62 proteins with the markers of the PCs and CN neurons, including LMX1α/Tbr1a and Foxp2/Ptf1a, respectively. Last but not least, the experiment can be designed by using the primary cell culture of the PCs and CN neurons to determine the distribution and localization of the TGF-β ligand and receptors to detect whether PCs and CN neurons are the source or target of the TGF-β. Using this method and to assess the response of PCs/CN neurons to TGF-β, PCs/CN neurons can be cultured in 3 different groups, including Control which is in the presence of DMEM-F12 medium, and treatment groups including (A) supplemented with TGF-β_1 and (B) the pharmacological inhibitor of TGFβRII (SB-431542). Then, levels of the PSmad can be analyzed to determine the responsiveness of the PCs/CN neurons to exogenous TGF-β_1. If treatment of PCs/CN neurons with TGF-β_1 increase the levels of P-Smad, this may suggest that these cells are responsive to TGF-β1. PCR would confirm results to see the expression of both the receptors and the factor itself to see if PCs/CN neurons are the target of the TGF-β_1 or the source. Then, we can visualize the colocalization of the TGFBRII and the ligand to confirm the presence of the pathway in the PCs and CN neurons.

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