THE PROLIFERATION AND MIGRATION OF GRANULE CELLS IN THE CEREBELLAR CORTEX OF NEONATAL SWISS MICE FOLLOWING MATERNAL TRYPTOPHAN ADMINISTRATION

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ABSTRACT

The External Granular Layer (EGL) of the cerebellar cortex is a region of proliferative cells which migrate to their last position in the internal granular cell layer of the cerebellum. Delay in the transit of cells from the EGL has been associated with deficiency of tryptophan, an amino acid, which is important in cerebellar maturation and in the disappearance of the EGL. The role of tryptophan in the development of the cerebellar cortex has not been fully looked at. In this study, the profile of the EGL in neonatal mice was examined following maternal administration of tryptophan.

Forty pregnant female Swiss mice weighing between 28 and 45g were allotted to four groups (n=10). The day the mice delivered was postnatal day (PND) 0. Control group (Group I) received distilled water, Groups II-IV orally received 100, 200 and 300mg/kg body weight solution of tryptophan, respectively. Dams in each group received a daily dose of tryptophan postpartum for 0,7,14,21 days and the pups (n=5) were sacrificed after the last dose. In the pups, Brain Weight (BW) and Cerebellar Weight (CW) were measured by a weighing scale; Brain Tryptophan (BT) and Serum Tryptophan (ST) levels were measured by spectrophotometry. The cerebella were fixed in 10% formalin, coronal sections taken at the paraflocculus were stained with haematoxylin and eosin for estimation of cerebellar cortical layer width, neuronal cell counts and mitotic index. Other coronal sections were stained with cresyl violet for radially oriented granule cells; modified Golgi stain for dendritic arborization of Bergmann glial cells; ki-67 immunostaining for cell proliferation and Bcl-2 immunostaining for apoptotic cells. Data were analysed using ANOVA at $\alpha_{0.05.}$

On PND7, BW of GroupII (0.25 ± 0.07g) was significantly greater than Group I(0.20 ± 0.03g), GroupIII (0.20±0.05g) and GroupIV (0.17±0.03g) and CW of GroupII (0.06±0.01g) was greater than GroupI (0.05±0.01), GroupIII (0.05±0.01) and GroupIV (0.03±0.07g). External granular layer width of GroupI (0.11±0.00mm) was less than GroupII (0.13±0.05mm), GroupIII (0.13±0.13mm) and GroupIV (0.13±0.02mm) on PND14. Percentage BT in GroupII (9±2x10^{-4}%) and GroupIII (12±2x10^{-4}%) was significantly higher than GroupI (8±2 x10^{-4}%) and GroupIV (7±2 x10^{-4}%). Serum tryptophan in GroupII (0.020±0.012%) was significantly higher than GroupI (0.005±0.002, GroupIII (0.011±0.005) and GroupIV (0.002±0.001%) on PND21. The EGL density of GroupIV (34.0±5.15mm^2) was higher than GroupI (23.6±8.02mm^2), GroupII (30.7±2.5mm^2) and GroupIII (31.4±2.5mm^2). Mitotic index was significantly higher in GroupIV (11.8%) than others (6.6,7.9,6.2%). Number of radially oriented granule cells was significantly higher in GroupII (2.6±0.62; 2.98±1.02), GroupIII (2.9±0.93; 2.3±0.42) and GroupIV (2.7±0.29; 3.1±0.86) than GroupI (1.67±0.48; 1.28±0.43) on PND7 and 14. The EGL persisted in GroupII pups compared to others on PND21 and there were more Bergmann glial cytoplasmic processes in GroupII than in other groups. Percentage positivity of ki-67 cells was significantly increased in GroupII and GroupIII on PND7 and 14. However, the percentage positivity of Bcl-2 was significantly increased in GroupII on PND7.

Low dose tryptophan prevented the early loss of cells in the EGL which can be a potential site for adult neurogenesis and a source of stem cells.

Keywords: Tryptophan, Cerebellar Cortex, External granular layer, Proliferation, Migration

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I thank GOD for the grace to accomplish this work.

DEDICATION

This is dedicated to all those who matter so much to me and contributed in no small measure to the completion of this work.

CERTIFICATION

I certify that this work was carried out by Olubunmi A. Onatola under my supervision in the Department of Anatomy, University of Ibadan.

……

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ACRONYMS

ANOVA	Analysis of Variance
BBB	Blood Brain Barrier
BC	Basket Cell
BG	Bergmann Glia
CC	Cerebellar Cortex
CP	Cerebellar Plate
CN	Cerebellar Nuclei
CNS	Central Nervous System
DAB	$3,3^{1}$ – Diaminobenzidine
DCN	Dorsal Cerebellar Nucleus
DNA	Deoxyribonucleic Acid
dWM	Deep White Matter
E	Embryonic day
EGC	External granular cells
EGL	External Granular Layer
EGZ	External Germinative Zone
GC	Granule Cell
GCP	Granule Cell Precursor
GoC	Golgi Cell
GM	Gray Matter
H & E	Hematoxylin and Eosin
IGL	Internal Granular Layer
IHC	Immunohistochemistry
L-TRP	Tryptophan
ML	Molecular Layer
PC	Purkinje Cell
PCL	Purkinje Cell Layer
PND	Postnatal Day
pWM	Prospective White Matter
NOND	Naturally occurring neuronal death

RGZ	Rostral Germinative Zone
SC	Stellate Cell
SVZ	Subventricular Zone
TDO	Tryptophan-2, 3- Dioxygenase
UBC	Unipolar Brush Cell
uRL	Upper Rhombic Lip
VZ	Ventricular Zone
WM	White Matter

CHAPTER ONE
INTRODUCTION

1.1 Background

Development of the central nervous system (CNS) has many phases involving cell multiplication, differentiation and movement, cell-cell contacts and formation of synapses (Sotelo, 1990). The growth of the CNS is decided principally by genes, and it is also influenced by the external and internal milieu. Cells in the maturing CNS are produced in particular zones that are germinative and reach their final position through active migration. The movement is assumed to be controlled partially by genes and a distinct set of events indicated by the cells that migrate. All through the early period of proliferation, all external granular cells (EGCs) lie in the nascent germinal layer and these seem headed for involvement in Deoxyribonucleic Acid (DNA) synthesis and somatic cell division (Trenkner *et al*., 1984).

The cerebellum has an exceptional cytoarchitecture made of five separate layers: external granular layer (EGL), molecular layer (ML), Purkinje cell layer (PL), internal granular layer (IGL) and white matter (WM). Purkinje cells (PCs), interneurons, and granule cells (GCs) lie in the PCL, ML, and IGL sequentially. Apart from these cells, the cerebellum, in addition contains different glial cells in the various layers: Bergmann glia (BG) in the ML and PL; fibrous astrocytes in the WM, protoplasmic astrocytes in the IGL, and oligodendrocytes in gray matter and WM. These neurons are different in structure and function (Jacobson, 1991). These neurons in the developed cerebellar cortex (CC) and nuclei are produced from the early production and movement of precursor cells which transform into various components of the cerebellar nuclei comprising PCs and Golgi type II neurons (Jacobson, 1991).

Additionally, a proliferative zone lies below the pia, identified as the EGL, evident in later stages of embryonic development and persists throughout early post-natal life in mammals, it differentiates to cells of the IGL namely: basket cells (BCs) and stellate cells (SCs) (Ryder and Cepko, 1994). The entire procedure is controlled by a sequence of spatial and molecular connections among the neurons that migrate and by twigs of the radial glia (Sotelo *et al*., 1994). In the maturing cerebellum, the increase in the quantity and transformation of glial cells along with neurons rely on the signaling pathway known as the sonic hedgehog (shh) pathway (Marrazziti *et al.,* 2013).

Tryptophan (Trp) is a protein unit that is not manufactured in the body. It is required for daily nutritional consumption. It is unique because of its diversity of biological roles (Moffat*et al.,* 1986) and an important compo`nent of protein metabolism in human nutrition. Essentially, Trp is antecedent to the neurotransmitter, serotonin, which partakes in the functioning of the brain and other associated controlling roles (Liang and Song, 2005). It is useful in the treatment of schizophrenia, depression and high blood pressure (Budaveri *et al.,* 1996).

As a unit of protein that is needed in the diet, Trp is vital in the synthesis of protein and its function in cellular differentiation, development and rate of the CNS is essential. Monoamines affect quite a number of cellular events early in life because of their paracrine role in neurogenesis, movement and growth of cells, cell differentiation, formation of synapses, regression and cell death that is predetermined (Soto-Moyano *et al*., 1998). A mitogenic substance *cum* amino acid that is capable of inducing cell division is tryptophan which participates in biological functions, such as protein and energy syntheses, control of the immune system and higher functioning of the brain (Kanai *et al*., 2010).

Tryptophan is associated with the growth and maturation of the cerebellum plus its related cognitive and behavioral functions. Tryptophan is also assumed to be involved when cerebellar maturation begins. Tryptophan-2, 3-dioxygenase (TDO) – an important regulatory enzyme needed for Trp metabolism pathway, helps in adult neurogenesis by regulating the growth of new cells within the hippocampus, olfactory bulb and subventricular zone (SVZ) (Funakoshi, 2011). Slow growth rate of Bergmann glial cell (whose somata is found alongside Purkinje cell somata in the PL) maturation occurs when Trp is deficient in the diet before fertilization (Del Angel-Meza *et al*., 2001).

Cerebellar cortex (CC) is the grey matter arranged mostly as a thin stratum casing the central part of white matter. Contrary to the cerebral grey matter, all regions of the cerebellum have three layers and the CC has a uniform structure.

The cerebellum is roughly ten percent of the whole brain mass. When not folded, the surface area of CC is partially half of the cerebral cortex. The CC contains less dissimilar cell types which unlike the cerebral cortex has numerous cell types located differently in various regions and are organized in a highly fixed way and appear similar in histological sections.

Discrete cells are located in the cerebellar gray matter which include basket, outer stellate, Golgi, Purkinje, granule, brush and Lugaro cells. A few glial cells are found which include astrocytes

and Bergmann glia. Granule cells are the most copious and they synapse with fibers within the cortex especially mossy fibres. Bergmann glia are scaffold that assist in the movement of GCs to the EGL.

1.2 Statement of the Problem

An adequate understanding of the activities that take place in the nascent cerebellar cortex remains a challenge. Malnutritition or amino acid deficiency can induce morphological and functional alterations in brain development. According to the life stage affected, this can occur by affecting the number of cells formed, by influencing cell migration, by delaying or blocking cell growth and differentiation, or by increasing cell death (Levitsky and Strump, 1995).

Neuronal and glial differentiation and synaptogenesis depend on the brain region involved and its critical developmental periods. Cerebellar maturation takes place postnatally, and it has been reported that the cerebellum is more sensitive to a wide range of adverse environmental factors than the rest of the brain during its early postnatal development (Hillman and Chen, 1981).
Experimental studies carried out on the incremental cellular quantity and movement in the cerebellar cortex have been explored, but the role of Tryptophan, which could accelerate cellular proliferation and migration, is yet to be explored. It is, therefore, pertinent to examine the production of new cells and movement of these cells especially during the postnatal phase in the presence of Trp.

1.3 General Objectives

To study the progression of gross morphology, structural changes, and cellular activities which occur postnatally in the cerebellar cortex in relation to the duration of Trp administration.

1.4 Specific Objectives

1. To examine the proliferation of cells and the mitogenic properties of tryptophan in the young cerebellar cortex
2. To study the outcome of Tryptophan administration on the mode by which GC migrates in the cerebellar cortex
3. To examine the level of pyknosis and regulated cell death in the newly formed cerebellar cortex
4. To highlight the implication of Tryptophan dosage on Bergmann glial maturation.

1.5 Justification

The EGL in the cerebellum is a secondary germinal layer which is known as the source of the three categories of cerebellar circuit neurons that are confined within the layer: BCs, SCs and GCs. Tryptophan has been found to have a neuroproliferative ability in different brain regions (Kanai *et al*., 2009). One site that is identified for postnatal neurogenesis is the EGL of the cerebellar gray matter (Obersteiner, 1883). Tryptophan may further potentiate neurogenesis and plasticity in the EGL in the developing brain of mammals by stimulating active cell division in the EGL. Granule cells that survive harmful effects can proliferate and reconstitute the EGL. This may be of benefit in stem cell research, neuroprotection and transplantation of organs.

This study is expected to increase the understanding of the way administration of Trp affects the density and movement of the GCs in the cerebellum after birth. This is important because structure and function are related. Alteration in the orderly organization of the neurons during a crucial period of the development of the cortex may produce considerable later consequences.

Studying the developmental profile of the granular neuronal dendrites in the EGL will enhance the understanding of the mode of expansion present in the CC.

1.6 Scope of the Study

This research involved Trp administration to postnatal mice. Changes in brain and cerebellar weight were examined in the mice which were sacrificed for gross assessment of the cerebellum. Cerebellar tissues were processed and stained for quantification of neurons, especially granule neurons. Immunohistochemical staining was done to determine neuronal morphology.

CHAPTER TWO

LITERATURE REVIEW

2.1 CEREBELLUM

The cerebellum is implicated in the co-ordination of locomotion, it is a major part of the brain that does this function of moving the body parts. It is made up of a well arranged layered cortex which consists of gray matter (GM) superficially and a medullary body located deep to the gray matter, composed of white matter that extends to create a distinctive arborization on the axis of each folium of the cerebellum. Three (four in humans) cerebellar nuclei (CN) of neurons exist from which a number of the cerebellar efferent fibers arise (Cocito *et al.*, 2015).

The cerebellum performs a crucial function in managing carriage and intended movements. Reflexively, it controls the voluntary muscle contraction effectively and carefully harmonise their actions. Posterior cranial fossa houses the cerebellum and is roofed by tentorium cerebelli. This is the main structure in the hindbrain and is situated behind the the pons, medulla oblongata and fourth ventricle. Cerebellum is oval and constricted in the middle, with two cerebellar hemispheres joined by a slender vermis in the middle. Three major collections of fibers of nerves namely: superior, middle, and inferior cerebellar peduncles link the cerebellum to the hind portion of the brainstem (Voogd *et al*., 1996).

Cerebellum possesses three lobes: the anterior, middle, and the flocculonodular lobes. The anterior lobe is placed on the upper portion of the cerebellum delineated from the middle part via a broad fissure that is V-shaped known as the primary fissure. The middle lobe otherwise known as posterior lobe is the biggest division. It is positioned amid the primary and uvulonodular fissures. The flocculonodular lobe is behind the uvulonodular fissure. A deep horizontal fissure lies on the entirety of the periphery of the cerebellum and demarcates the first part from the lower surfaces; and this has no morphologic or functional value (Voogd *et al*., 1996).

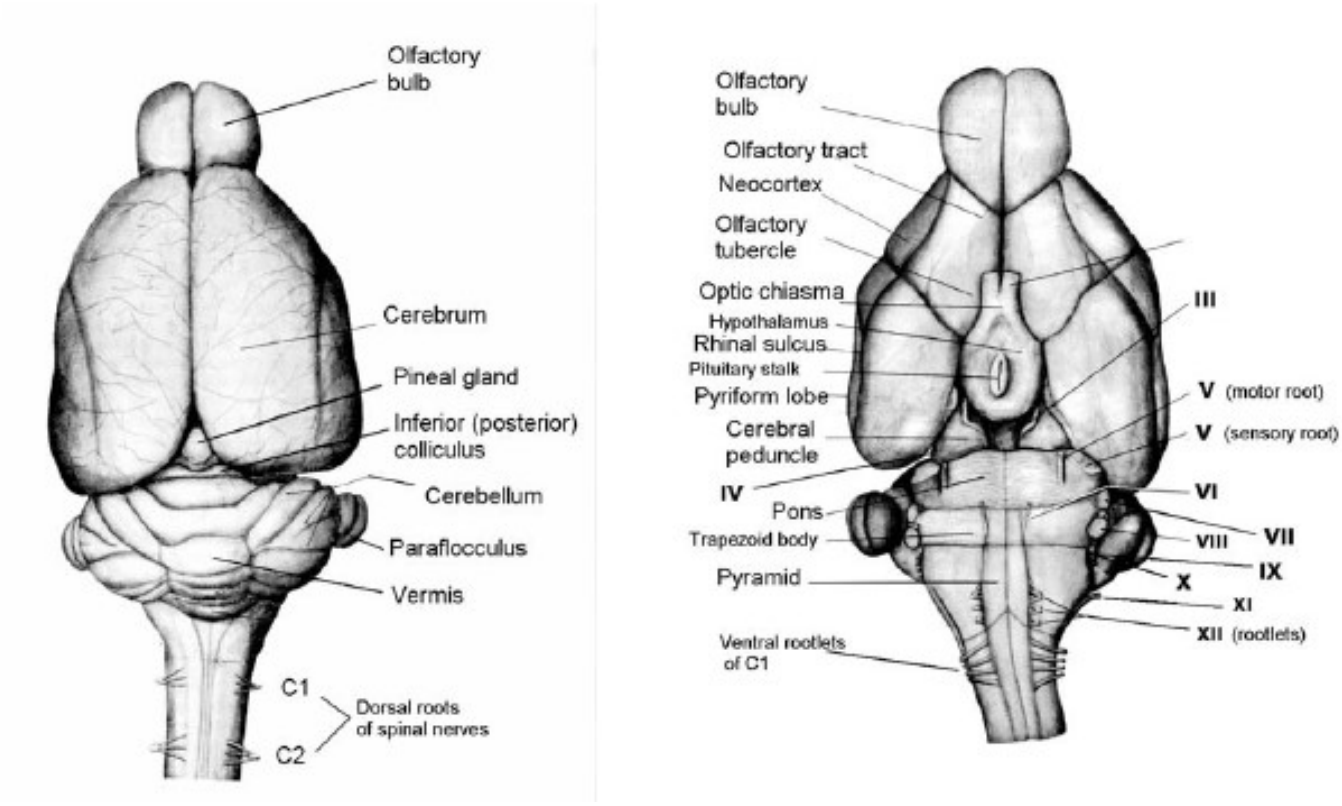
Within the cerebellum is an outer GM also identified as the cortex and inner WM. Enmeshed in the WM of either hemisphere are three collections of GM which forms the cerebellar nuclei. Three layers of cells are found within the cerebellar cortex which consists of components of neurons including outer SCs and BCs in the ML, within the PCL are Purkinje and candelabrum cells; and Golgi cells (GoCs), GCs, Lugaro cells and unipolar brush cells (UBCs) are available in

the IGL. Cellular components of the CN are sited near the top of the fourth (4^{th}) ventricle in the cerebellar WM. These nuclei and other vestibular nuclei make up the major cerebellar output (De Zeeuw and Berrebi, 1995).

The normal structural arrangement of the cerebellum is classified into four horizontal types depending on the genes present and the termination of afferent fibers. The lobe and lobule divisions do not align completely with the boundaries of these zones, but give a more useful way of separating the cerebellum into its various parts (Marzban *et al*., 2014). Of all the CNS structures, the cerebellum consists of many circuits which are highly patterned and vital in the arrangement of many functional and topographic circuits located in various zones. Researches on molecular heterogeneity have revealed the manner whereby genes may be expressed in cerebellar nuclei neurons which may reflect how complex the cerebellar cortex is. As development begins, vital cerebellar structure is laid down and comes before the events of axonogenesis and neurogenesis, at this time circuits of the cerebellum and functions are already recognized.

The cerebellum is partitioned into lobes by fissures and it is made up of a highly structured set of folia and as such is a distinct structure of the CNS. Lately, it was assumed that the cerebellum takes part in executive functions which includes emotion, cognition and acquisition of language skills not excluding its established role in synchronizing proprioceptive-motor activities (Tavano *et al*., 2007). Neural control of muscular movement is performed by the cerebellum. The unique organization of the layers is fundamental to cerebellar function. In utero, granule cell precursors (GCPs) populate the EGL under the glia limitans which is produced by end feet of BG and travel on its fibers to enter the IGL.

Surface area was increased when folia was introduced to the cerebellum during evolution and this allows for a rise in number in the population of cells, which will assist the acquisition of more intricate functional circuits (Welker, 1990). Three discrete cell layers are found in each folium which surrounds the white matter and deep nuclei. Purkinje cells produce sonic hedgehog (Shh) which control the number of folia by influencing the GCP proliferation (Corrales *et al*., 2004; 2006). Contrarily, postnatal expansion phase of folia is reliant on the disparity in the different speeds at which GCP proliferates, maximum speed being in the inferior part of the partitions (Mares, 1970).



a. b.

Figure 2.1a: The dorsal surface of the rodent brain and upper two divisions of the spinal cord, b: Ventral aspect of the brain, and junction of medulla with spinal cord. (Source: ratpix.pdf, 2008)

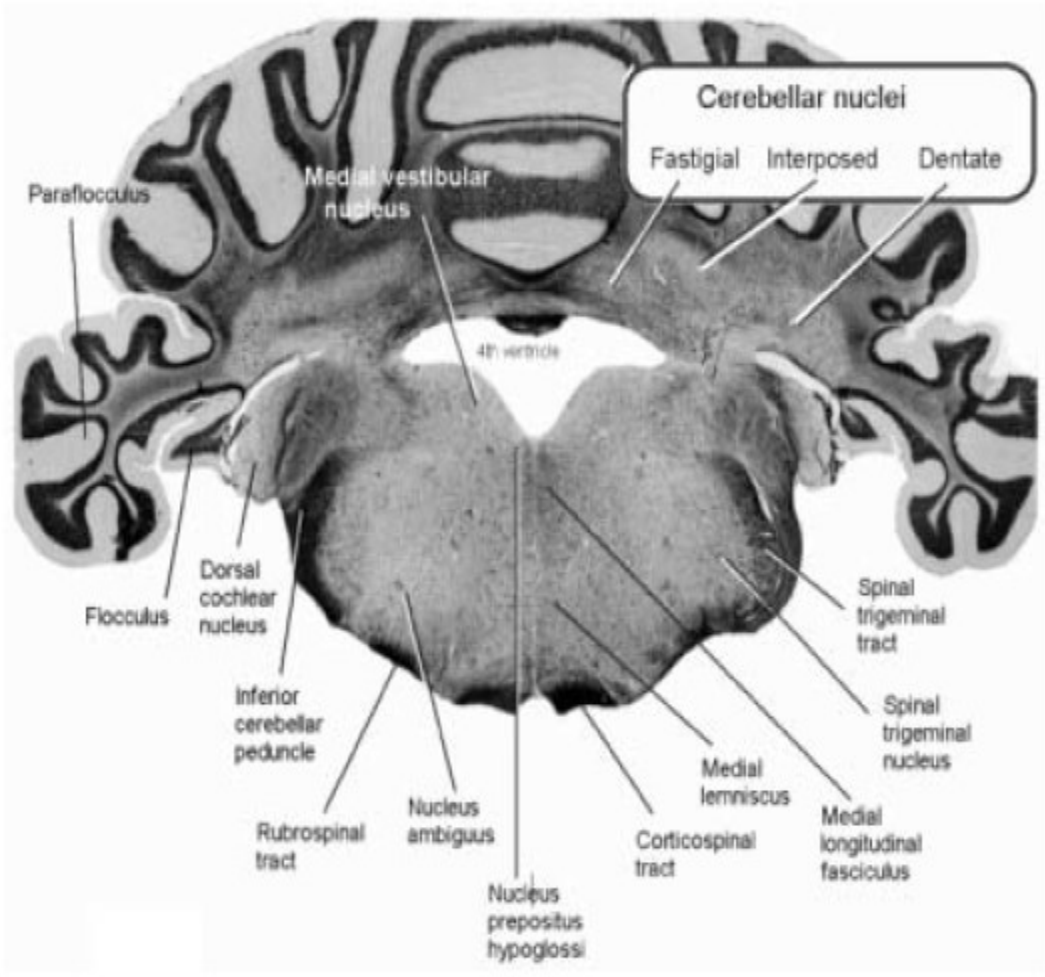


Figure 2.2 Transverse Section through the paraflocculus. (Source: Ratpix.pdf, 2008)

Cerebellar cortex is prearranged into four layers as seen during development, this include the EGL, ML, PCL and IGL (Komuro & Yacubora, 2003). The aminobutyric acid (GABA)ergic PCs are the lone cells that project out of the CC and are found located in the PCL amidst the EGL and the IGL at the time of birth. In the mice, the single layer display of Purkinje cells reaches completion on postnatal days 4-5 (PND 4-5) (Yuasa *et al.*, 1991). The EGL disappears after 21 days after birth, by then, the IGL is already prearranged and successful cerebellar maturation has ended (Maricich and Herrup, 1999).

The cerebellar cortex is a good region useful in studying the primary principles of brain growth, function, organization, and disease considering the little quantity of neurons that make up the structure of the cerebellum (Sillitoe and Joyner, 2007). In the cerebellar GM, the GC is the most important neuron, making up almost half of all the cellular varieties found in the CNS. In the upper rhombic lip (uRL) are formed the GCs of the 4th ventricle within the proliferative layer (Alder *et al.*, 1996). Migrating from the uRL at about the 13^{th} day of embryogenesis (E13), the GCPs travel superiorly on the periphery of the cerebellar anlage to establish a portion where mitosis is active known as the EGL as observed in mice. On E15, the EGL is formed and GCPs within the EGL are actively dividing up till the third week after delivery (Hanaway, 1967). Few GCPs begin to egress the cell cycle at the period of birth, and get transformed into fully grown GCs leading to the continous dissolution of EGL. Initially, GCs stretch their axons opposite the pial surface at first and moves radially on the length of BG processes to settle down in the IGL, the ultimate location of GCs (Adams *et al.*, 2002). Present in the PCL are cell bodies of BG and these spread their single processes to reach the pial matter and are essential for formation of laminae in the cerebellum. The IGL can be seen on PND 5 to become absolute by PND 20 (Altman and Bayer, 1997).

Up to six separate kinds of interneurons are derived in the two vital zones with increased multiplication of cells— uRL and the cerebellar plate ventricular zone (VZ) — which form in sequence (Fahrion, 2013). From the uRL or the VZ most interneuron precursors migrate out to a second zone where similar multiplication occurs, the EGL, the deep white matter (dWM) or the prospective white matter (pWM) (Fahrion, 2013). Here, the cells move to their ultimate location in the ML or in the IGL in the first 21 days after birth. Originating from the uRL are two different kinds of interneurons who are actively firing— the UBCs and the granule cells. Whilst GCPs migrate to the EGL, UBCs move onwards to the IGL through the GM. In the mice, a large

number of UBCs get to the IGL by PND 10, while the last GCs arrive at their ultimate location in the IGL on PND 20 (Komuro *et al.*, 2013).

The other four cell types are interneurons that are inhibitory — BCs, the SCs, Lugaro cells and the GoCs – are derived in the VZ (Komuro *et al*., 2013). Golgi cells travel across the dWM past the folia of the WM to the IGL till PND 4 in mice. Stellate and basket cells also move from the dWM across the folial WM, IGL and the PCL to the settle in the ML until PND 16 in mice. Lugaro cells travel across the WM of the folia to their utimate destination superior to the IGL by PND 5 (Hsu *et al.,* 2003).

Granule cells employ centripetal migration while the other cell types: UBCs, SCs, BCs, LuCs and GoCs use centrifugal migration. In the cerebellar cortex this allows for an excellent model to study migration postnatally. In the three preliminary weeks following delivery in the rat, the GCs are the most numerous migrating interneurons followed by BCs / SCs with levels of compactness of 1124 ± 138 and 306 ± 79 cell/mm^2 respectively on PND 10 (Raoult, 2014).

Interneuron	Role	Primary Germinative Zone	Secondary Germinative Zone	Final Destination	Completion of Migration*
Granule cell	Excitatory/ Glutamatergic	uRL	EGL	IGL	P20
Stellate cell	Inhibitory/ GABAergic	VZ	pWM	Top ML	P16
Basket cell	Inhibitory/ GABAergic	VZ	pWM	Bottom ML	P16
Golgi cell	Inhibitory/ GABAergic	VZ	pWM	IGL	P4
Unipolar brush cell	Excitatory/ Glutamatergic	uRL	--	IGL	P10
Lugaro cell	Inhibitory/ GABAergic	VZ	dWM	Top IGL	P5

Table 2.1: Features of interneurons in transit in the cerebellum afterbirth. * In mouse or rat. (Source: Galas *et al.*, 2017).

2.2 DEVELOPMENT OF THE CEREBELLUM

Early cerebellar structures contain parent neurons from which the other cells originate and are arranged in three unique areas: fourth ventricle neuroepithelium, the uRL and the cerebellar WM. Components of the CN, the PCs, and interneurons apportioned for the IGL are all formed in the neuroepithelium of the 4th ventricle. Molecular layer cerebellar interneurons are produced from precursors who have glial features in the interlobular and dWM (Silbereis *et al*., 2009). In the mouse, contrary to GCPs which travel to the cerebellar primordium from the uRL, extending tangentially along its periphery as the EGL at delivery and staying till PND 20, they exit all events leading to division of cells and travel along the processes of the BG to inhabit the IGL (Komuro *et al.*, 2001).

In most mammals, a greater part of the developmental changes in the cerebellum occur afterbirth (Altman and Bayer, 1997). The gradual enhancement in size of the CC after birth is composed of a wide array and a chain of events that are complex which culminate in the production of the GABAergic cortical interneurons and granule cells. Neurons and glial cells move very long distances from the site of their progenitors and spread their axons to make exact synapses with target neurons (Marzban *et al.*, 2015). Neurogenesis taking place in the EGL gives rise to GCs that move through the ML and the PCL to later get to the IGL, which is the sole granular layer in the adult CC (Altman, 1972).

The cerebellum is a unique part of the brain and extends halfway without indications of the structural basis of the location of where the right and left halves unite. It is derived from two discrete embryonic anlages which are formed in the upper part of the fourth ventricle. As the young anlage approaches each other they meet on embryonic day 15 in the mouse and almost totally unite in the next 48 hours. Different neurons: Purkinje cells, GCs and the neurons of the deep cerebellar nuclei (DCN) which are the essential types of neurons have been previously studied and documented (Altman and Bayer, 1985). Rhombic lips form when the dorsolateral areas of the alar plates bend in the middle and are widely separated in the caudal portion of the metencephalon, but they approach each other in the midline directly below the mesencephalon.

As the pontine flexure deepens further, the cerebellar plate is formed when the rhombic lips are compressed cephalocaudally. In human, the cerebellar plate indicates a little part in the middle, the vermis, and two parts by the sides, the cerebellar hemispheres in an embryo aged 12 weeks. A horizontal line partitions thevermis from nodule and the hemispheres from the flocculus. The

most primitive segment of the cerebellum is the flocculonodular lobe. In the onset, the cerebellar plate is composed of neuroepithelial, marginal and mantle layers. As there are gradual changes in development, a large proportion of neurons derived from the neuroepithelium move to the exterior part of the cerebellum to become the EGL. Within this layer, neurons conserve the capacity to get fully involved in cell division and a zone where proliferative activities take place is formed on the exterior of the cerebellum (Altman and Bayer, 1997).

About Embryonic (E) day 7–8, on the apical lip of the fourth ventricle, the mouse cerebellar primordium appears as a neuroepithelial swelling which is a fraction of the metencephalic alar plate.

The cerebellum in the rat initially appears as double condensations of the metencephalic RL on E11–12 (Altman and Bayer, 1997). Two distinct germinative layers are responsible for histogenesis within the developing cerebellum: ventricular neuroepithelium and EGL. The initial neurons to be formed in the neuroepithelium are the PCs and cells of the CN. Peak formation of these cells occurs on E13–14, and ceases by E16 excluding few PCs (Altman and Bayer, 1978). On E19, GoC production in the neuroepithelium starts and persists for few days after birth. From the 16th day of embryogenesis, the EGL has started spreading from the germinative trigone to the exterior of the maturing cerebellum.

Afterbirth, neurons found in the EGL go through immense multiplication to produce GCs - the most plentiful neurons in the cerebellar cortex (Altman, 1972). Majority of interneurons available in the cerebellum are the SCs and BCs which are produced after birth from precursors that are different from those in the EGL (Zhang and Goldman, 1996). These neurons are the ideal model with which to explore structural origin in the CNS because of the existence of the few quantities of well-defined cells in the cerebellum.

During the sixth month of human development, the EGL produces different cell types which move toward the maturing PCs and whereby GCs are formed. Proliferating cells in the cerebellar WM produce basket and stellate cells. Cerebellar cortex which consists of Golgi II neurons, PCs and neurons produced by the EGL, reaches its ultimate size postnatally. Dentate nucleus which is one of DCNreaches its final location in utero.

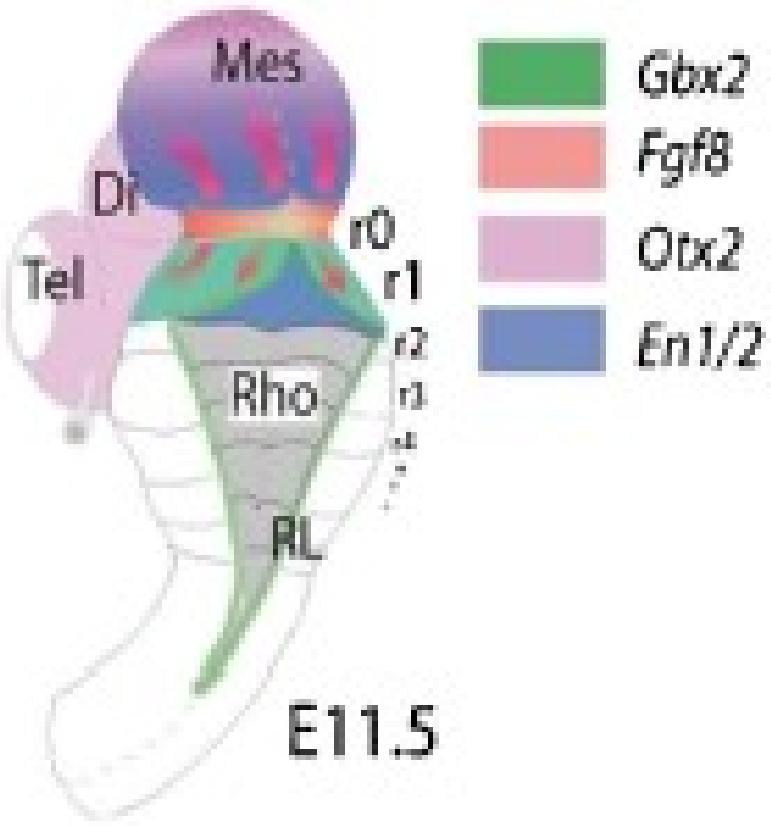
The cerebellum is divided by folds and fissures into lobes, lobules, and folia. Mammalian vermis all have a comparable fundamental blueprint of ten folia, proposing that folia are determined by genes (Cajal, 1911). Three different layers of cells surround the deep CN and WM in each

folium. The number of folia is controlled by Shh produced by PCs through its control on GC proliferation (Corrales *et al*., 2006).

Murine cerebellar growth relies on a carefully planned relationship among many signaling pathways, which regulate migration, multiplication and transformation of a few glia and nerve cells.

The isthmus is a thin ring which encircles the neural tube amidst the mesencephalon and rhombencephalon and forms the front border line of the primordium of the cerebellum. Isthmic organizer is housed in the isthmus and it is required for the maturation of the mesencephalon superiorly and rhombencephalon inferiorly. This organizer regulates different activities such as cell identity and survival of the cell reliant on the developmental phase. Inferior limit is the upper portion of the 4^{th} ventricle.

At birth, the cerebellum is not formed fully and major histogenetic events take place postnatally in the first three weeks (Inouye and Kameyama, 1983). Granule cells are derived in the uRL and migrate as a slim sheet on the exterior of the maturing cerebellar anlage, and divide without delay postnatally (Miale and Sidman, 1961). When proliferation commences, the totality of external granular cells (EGCs) is engaged in DNA synthesis and cell division. After the S phase, cells at once proceed from the periphery to inhabit the postmitotic zone in the innermost part of the EGL. Tagged neurons stay in the postmitotic zone for a day and then move into the deep parts of the WM in the mouse. The rest cerebellar cells seem to go through the same direction to enter the IGL and continously transit the cell cycle after the last mitosis. Granule cells spread throughout the IGL and use less than 5 hours to travel through the ML (Miale and Sidman, 1961).



Tel	Telencephalon
Di	Diencephalon
Mes	Mesencephalon
Rho	Rhombomeres
RL	Rhombic Lip
r	Rhombomeres

Figure 2.3: Posterior examination of a mouse embryo at the 11.5 day of embryogenesis showing the isthmic constriction positioned amidst the mesencephalon and the rhombomeres from which the cerebellum is formed. (Source: Leto *et al.*, 2016).

2.3 GERMINAL ZONES IN THE DEVELOPING CEREBELLUM

Primordium of the cerebellum is made up of two discrete germinal regions: the fourth ventricular wall neuroepithelium and the uRL (Englund *et al*., 2006). It was believed for a while that all cells of the cerebellum derive from the VZ, excluding GCs that are produced in the rostral lip. It is presumed that UBCs and nerves of the CN also have origin in the uRL (Machold and Fishell, 2005). A number of other germinal regions exists which are the rostral germinal zone (RGZ), external germinative zone (EGZ), and a portion of the cerebellar plate (CP).

2.3.1 *VENTRICULAR ZONE*

This zone produces all GABAergic cerebellar neurons (Hibi and Shimizu, 2012). Purkinje cells are formed in utero between 10^{th}–13^{th} day of embryogenesis in mouse (Hashimoto and Mikoshiba, 2003), whereas interneurons are formed after birth (Miale and Sidman, 1961).

2.3.2 *RHOMBIC LIP; CAUDOMEDIAL GERMINAL ZONE*

The uRL is found in all vertebrates as a region noted for high proliferation in the neural fold situated on the posterior rim of the 4th ventricle at the onset of CNS growth. It is further divided into eight parts: rhombomeres1–8, r1 is regarded as upper rhombic lip and r2–r8 regarded as lower rhombic lip), also quickly identified in the initial embryological phases in the posterior part of the brain (Altman and Bayer, 1997). The uRL was presumed to produce only GCs of the cerebellum (Altman and Bayer, 1997). From 12.5^{th} – 17^{th} day of embryogenesis, precursors are formed which travel to create the EGZ, which later produces the GCs within 14 days of life in the mice (Miale and Sidman, 1961). A number of glutamatergic neurons are obtained within the uRL; which comprise cerebellar nuclei neuron projection, which are formed at around 9th–12^{th} day of embryogenesis and the UBCs are formed from 13.5^{th} day of embryogenesis to few days after birth in mice (Hevner, 2006) and 15.5^{th} day of embryogenesis to few days after birth in rats (Sekerková *et al.,* 2004).

2.3.3 *EXTERNAL GERMINATIVE ZONE (EGZ)*

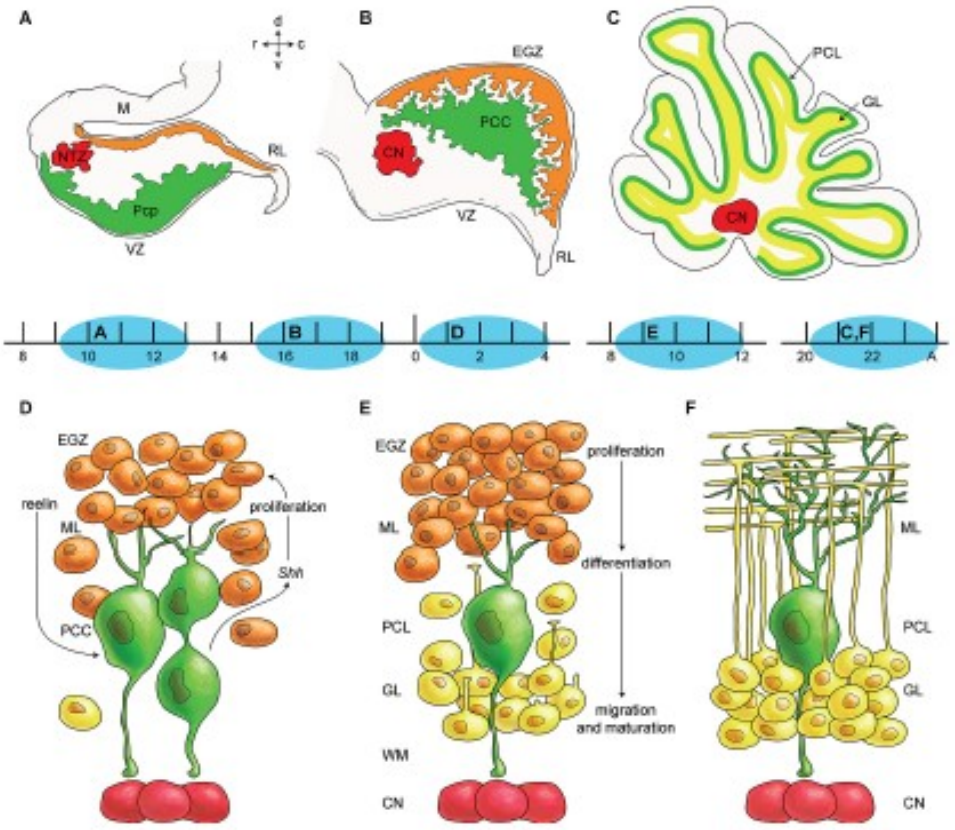
The EGZ is a transient group of cerebellar neurons that proliferate situated beneath the pia of the young cerebellum. The abundant number of neurons located in the EGZ produces GCs after birth. EGZ volume reaches a maximum on PND 15 and vanishes on PND 24. A few number of

GoCs are obtained from the EGZ. With bone morphometric proteins (BMPs) and Shh present, a minute percentage of GCPs transform into astroglia (Okano-Uchida *et al.,* 2004). Fastened to the lamina at the base of the outer demarcating sheet are GCPs in EGZ. The contact to the basement membrane is important because it amplifies the precursor cells in EGZ and may act in the same manner as the intermediary parent cells in the cortex in SVZ (Butts *et al.,* 2014).

The EGZ has two zones, which are: an external zone known for proliferation of cells and an internal zone known for cell division. The postmitotic GCs have axons which stretch amidst the parallel fibers in the nascent ML whereas the cell body moves internally to the IGL. Sonic hedgehog expression is needed in driving GCP proliferation and its secretion into the cerebrospinal fluid (CSF) enhances a rise in the quantity of parent cells and controls elongation of the rhombic lip and determination of cell fate. As development proceeds, shh engages the cells in transit in the WM. Detection of sonic hedgehogmRNA can be done in the Purkinje cells and EGZ at about the 17^{th} gestation week in the cerebellum of humans. The pia mater functions in GC proliferation and assists in the movement of GCs on Bergmann glial processes. As GCs develop, young branches form glomeruli in the IGL. This vigorous multiplication process is followed by apoptosis, as dead cells spread through the breadth of maturing cortex of the cerebellum (Silbereis *et al.,* 2010).

2.3.4. ROSTRAL GERMINAL ZONE (RGZ)

The rostromedial portion of the immature cerebellum develops commencing in the mesencephalon employing chimeras of quail-chicks. It was proposed that in the adult cerebellum, mes/rhombencephalon intersection during embryogenesis may be at lobule VI–VIII (Hallonet and Alvarado-Mallart, 1997). The RGZ is molecularly directed differently from those in the caudomedial germinative zone and performs a unique role in the maturation of anterior cerebellum.



M	Mesencephalon
VZ	Ventricular zone
RL	Rhombic Lip
CN	Cerebellar nuclei
ML	Molecular layer
PCL	Purkinje cell layer

WM	White matter
EGZ	External granular zone
Shh	Sonic hedgehog
Rho	Rhombomeres

Figure 2.4: Germinal zones in the maturing cerebellum (A-C). Sagittal sections in premature cerebellar development: A (E10–E13), B E16–E17, and C PND 20 to adulthood. Cerebellar cortex at PND 4 (D), at PND 10 (E), and in adult (F). (Source: Marzban *et al.,* 2015).

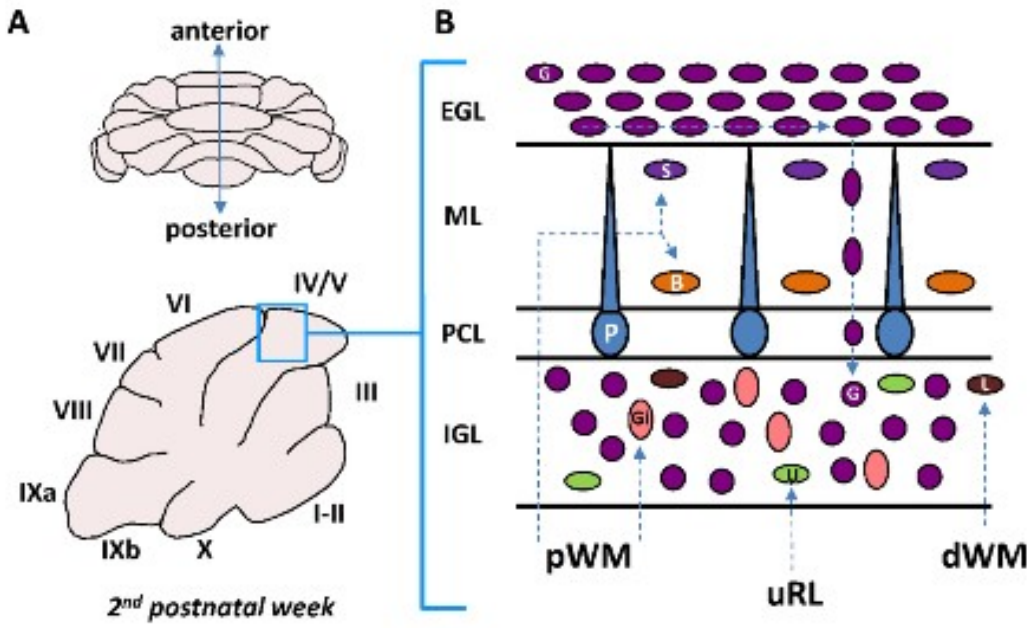
2.4 CELL MIGRATION DURING CEREBELLUM DEVELOPMENT

A vital cellular event necessary to structure the CNS is the migration of postmitotic cells from germinal zones. In premature cerebellum, cells are produced in many proliferative regions and move to their last station by employing radial or tangential pathways of movement. Bergmann glia are an unusual variety of radial glia (RG) located only in the cerebellum (Bellamy, 2006). These glia are derived from the VZ, and extend their apical processes underneath the pial layer and arrange their cell bodies side by side with the Purkinje cell body. Using electron microscopy, Bergmann glial fibers appear in the EGZ on the 15th embryonic day in mice and 17th day of embryogenesis in rat (Del Cerro and Swarz, 1976), and it appears in humans in the 9th week of conception (Choi and Lapham, 1980).

Tenascin histology using antibodies indicated radial glial fibers cross the whole mice cerebellum on embryonic day 13 (Yuasa, 1996). Human Bergmann glia expresses glial fibrillary acidic protein (GFAP) right from 12 weeks of gestation (Bell *et al.,* 1989). Purkinje cells with other precursor cells in the cortex that are inhibitory leave the cell cycle in the VZ in the mouse cerebellum, and they travel across the fibers of radial glia to create a platform for maturing neurons at the center of the anlage after the 13th embryonic day (Hatten and Heintz, 1995). Initially, the PC plate is thick but it later thins out into one layer at birth in the mouse (Xu *et al.*, 2013) and in 28th week of conception in human fetus (Zecevic and Rakic, 1976).

From the VZ are derived cortical inhibitory interneurons and these neurons progress to their terminal locations in the ML and the IGL. EGZ is established when proliferating cells that originate from rhombic lip travel rostrally to its site. Continous multiplication of EGZ cells creates a large collection of precursors that extends underneath the pia. Escaping the route of movement, the UBCs lie underneath the pia proximally next to the center of maturing cerebellum, whereas, large CN neuron projection and GCs travel continuosly to the distal part of the immature cerebellum in subpial stream pathway. The CN neuron precursors adjust course in the midway to get to the transitory zone. Precursors that are derived from the RL travel tangentially not depending on the guidance by glia in subpial route. Neurons in transit display a unique morphology whereby one leading process seems to direct the movement. Molecular mechanisms that make GCs change their manner of movement in tangential and radial phases are not clear. In the external zone of EGZ, there is granule cell precursor proliferation that occurs

less than 24 hours and cells that have divided stay between one to two days before the onset of their inward migration. In the period after cell division, the neurons migrate tangentially to the internal part of the EGZ and spread their transverse branches. The speed with which cells migrate is unhurried at the junction of the EGZ and the ML and this is followed by collection of dendrites into the ML. The GCs lengthen as they move radially on BG processes in the ML and passing through the ML between 10–11 hours to get to the PCL (Komuro and Yacubova, 2003). Neurons at once detach from the BG branches and their cell bodies are round. In the IGL, GCs travel radially to the base with no help from the glial cells. Movement is absolute as soon as the apex of an emerging twig reaches the WM.

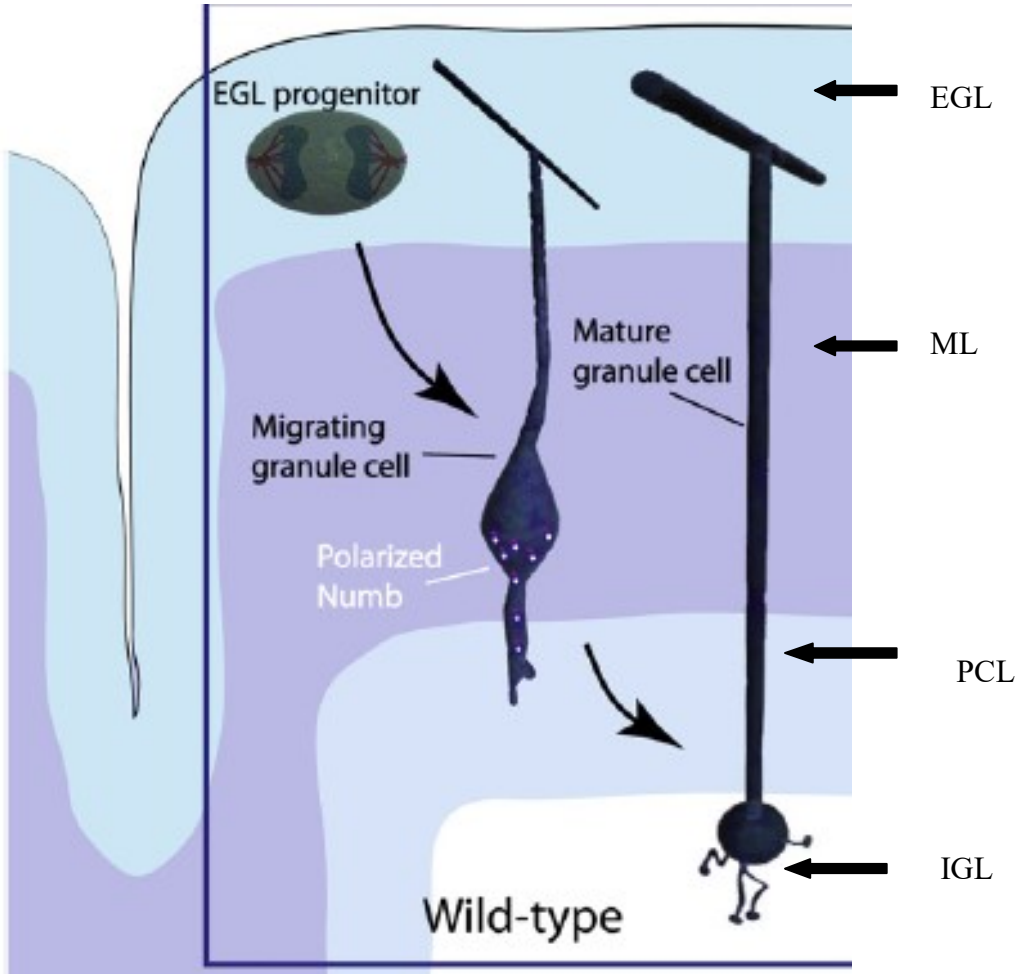


EGL	External granular layer
ML	Molecular layer
PCL	Purkinje cell layer
IGL	Internal granular layer
uRL	Upper Rhombic Lip
pWM	Prospective White matter
dWM	Deep White matter

Figure 2.5: The cortex of the mice cerebellum: (A) Cerebellum showing folia. (B) Migration of interneurons in mice: from germinative zones to final destination (Source: Galas *et al*., 2017).

Immediately, when mitosis is complete, GCs present in the cerebellum linger in the EGL between 1-2 days preceeding the start of radial movement of these cells in the ML, however, the importance of this rest phase is not very clear. With a confocal microscope, Komoro *et al*., 2001 examined the genes and morphological changes and activities of postmitotic GCs restrained to the EGL in microscopic slides of the cerebellum in neonatal mice. They observed coincidence in the length of the two unequal transverse processes placed in similar position to the elongated line drawn across the folium, as GCs that have undergone division start tangential migration in the route of the longer process. The speed, position and structure of cellular migration change in the EGL. Tangential speed of cell movement is very fast at 14.8 mm/hr at the centre of the EGL, with the neurons having double transverse processes that are small. As GCs lengthen their cell bodies and spread their elongated transverse branches to the base of the EGL, the neurons progress at a slow pace (12.6 mm/hr). Boundary of the EGL and ML allows cells to travel tangentially at a very slow speed (4.1 mm/hr), with oval cell bodies and the descending processes of the cells reach the ML.

Granule cells send one straight process and begin the change from tangential to radial mode of cell transit, whereby the shape of rounded cell bodies changes into a straight and long spindle after the stationary period. The mechanisms of development for the cells' correct positioning along parasagittal sections of the growing cerebellar gray matter may be provided by the tangential migration of GCs in the EGL.



EGL	External granular layer
ML	Molecular layer
PCL	Purkinje cell layer
IGL	Internal granular layer

Figure 2.6: Coordination of Cerebellar Granule Cell Migration in mice (Source: Breunig and Rakic, 2011).

In the EGL, morphological transformation and translocation of cerebellar GCs provides a chance to examine the fundamental processes of the movement of neurons and differentiation. From the lateral portion of the rhombencephalon does this secondary proliferative layer arise and covers the total surface of the cerebellar hemispheres before birth. This is corroborated by previous studies which using 3H-thymidine labeling supported the well acclaimed impression, suggested in 1911 by Cajal, that cerebellar GCs are produced in the EGL and that, subsequent to the last cells have divided, radial movement along the ML to the IGL occurs here, and where they finally settle in the adult cerebellum (Fujita, 1967).

In living slice preparations, real-time observation shows that after the terminal mitosis, future GCs stay behind in the EGL within 24 - 48 hours. At this time, many of postmitotic cells are arranged horizontally with their two transverse processes transformed into parallel fibers, as substantiated by the Golgi stained slide preparations of Cajal in 1911. It was observed that considerable tangential cellular migration occured in the period when the final division of these cells happened and at the start of their moving to the ML. The shape of the cell, mode, and tempo of migration were exposed clearly in the slice preparations and these changes carefully with respect to their location within the EGL.

Initially, the postmitotic cells are round and they send two small unequal transverse processes, arranged in a corresponding manner to the imaginary line drawn across the folium to be formed in the mid part of the EGL, which is located beneath the proliferative cell layers and these cells begin to move tangentially at a very fast speed. Base of the EGL allows GCs to attain fusiform shaped cell body and further extension of the two transverse processes is permitted while migrating with a moderately low speed. At the boundary of the EGL and ML, the movement of GCs is reduced significantly just before acquiring the third straight dendrite that will penetrate the ML that is developing radially. On the average, distance moved by GCs migrating through the tangential route will be more than 220 mm, considering that each cell may possess a substantially extended trajectory assuming that GCs that have divided use the same time on every level and that they do not overturn appreciably the route of their journey. 3H-thymidine studies report that the period of passage of these GCs that have divided in the EGL is on the increase in the initial period after birth (Rakic, 1985). This suggests that GCs that are produced later move further away from where they were derived in the course of tangential migration in the EGL than those GCs that were generated earlier.

Migration of neurons resulting from cell division from their point of origin is a basic activity necessary to build the CNS (Wingate, 2001). As cerebellum matures, cells are produced in a number of germinal zones and they move to their last spot by radial or tangential migratory routes. In the cerebellum, BG are a special type of radial glia. They are produced in the VZ, and they send apical processes beneath the pial layer and arrange their cell bodies close to those of the PCL.

Changes in the position of the cell beginning at the centre to the floor and to the EGL–ML margin seems to be due to weak dislodgment of the cells as revealed by electron microscopy (Rakic, 1971). The EGL exterior enlarges speedily on the outside owing to the fast division of cells of the GCPs after 20 hours severally in the first two zones of the EGL (Fujita, 1967). The EGL–ML border extends to the layer of pia as the soma exits the region of the EGL and their initial transverse branches change into parallel fibers, and then the ML had an increased length. Within 24 hours, GCs that migrate tangentially which are situated in the mid point of the EGL reposition to the EGL–ML margin. This occurs not involving an upturn in the strong descending migration of GCs. It was suggested that cellular substrate may be the cause of the tangential cell migration in the EGL but this is not proved yet. The fundamental cellular process is comparable to the tangential movement of developing nerve cells in the pons and medulla that are regulated by relationship amidst the migrating cells and neurites of old neurons (Ono and Kawamura, 1990). To buttress this hypothesis, microexplant cultures which showed that granule cells travel along several parallel fibers produced by early GCs has been reported (Komuro and Rakic, 1996).

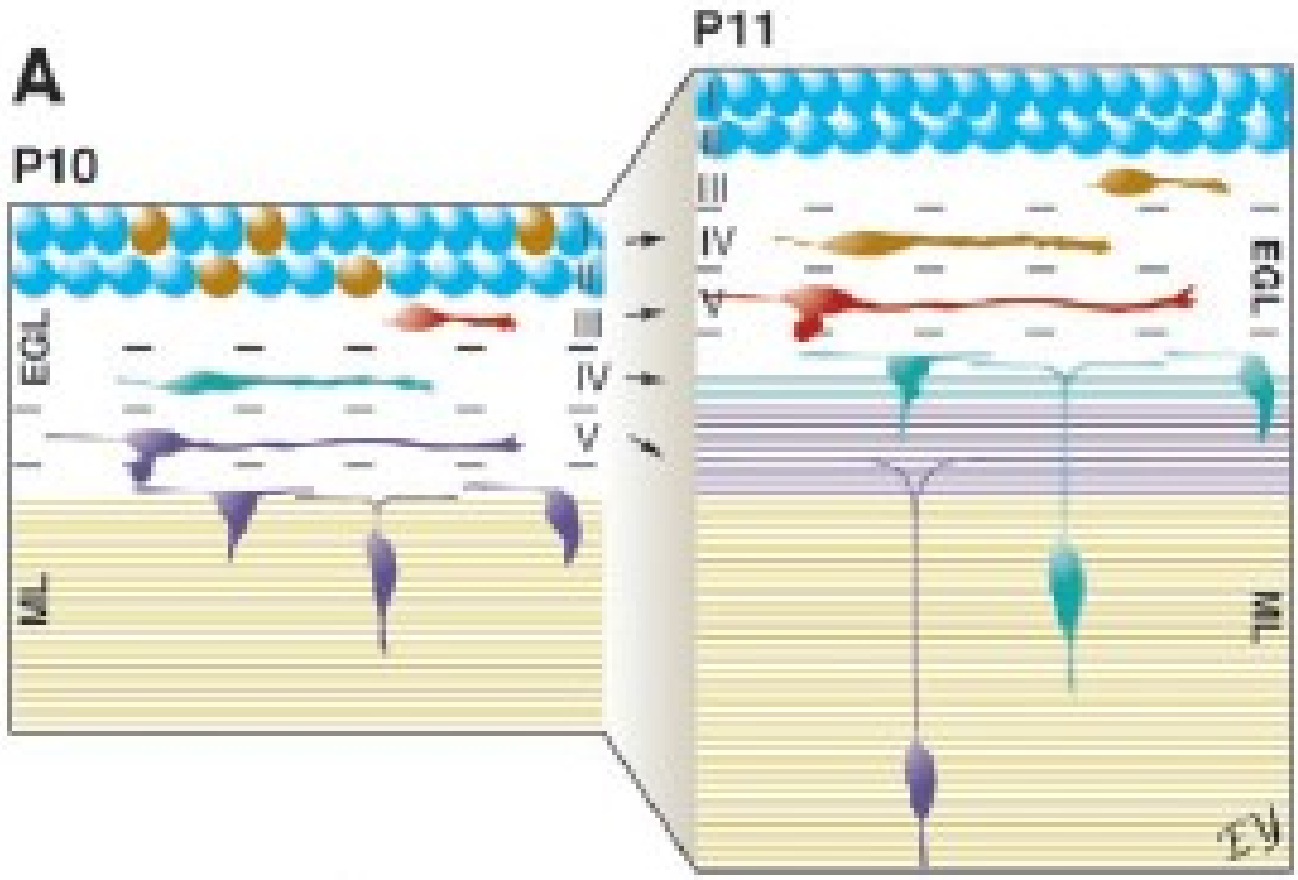
This manner of transiting similar to the type found in GC is the neuronal precursor tangential movement to the olfactory bulb from the SVZ in the telencephalon, which takes place using a sequence of migratory activities and avoiding the assistance of axonal processes or radial glia (Wichterle *et al.,* 1997). Another option is that the tangential migration of GCs may be chaperoned by gliophilic relationships at several links with the straight Bergmann glial processes as was proposed for the cortical neurons that migrate tangentially in the developing cerebrum (O'Rourke *et al.*, 1997). It is a possibility that at the EGL–ML margin the interaction of the downward processes of GCs that travel tangentially is abhorred by the PC dendrites.

Molecular mechanisms regulating the behavioral pattern of postmitotic GCs in the EGL have not been properly proved. As proposed, the transitory route of these cells might be due to the

molecules of the extracellular matrix (ECM) present on the surrounding molecules (Rivas and Hatten, 1995), as seen in other organisms. A number of molecules within the ECM are produced in the EGL (Chuong, 1990), and single GC migrates largely on the extracellular matrix molecules without axonal or glial processes. In the EGL, netrin family units are reported to be implicated in guiding tangential movement (Alcantara *et al.*, 2000). On the contrary, instigation of radial movement might rely exclusively on neuron–glia cell adhesion molecules (Ng-CAM). This is due to the fact that addition of these molecules may inhibit the repositioning of GCs that were tagged from the EGL to the ML (Chuong *et al*., 1987) but GC movement on the glial processes in cultures that are dissociated was not altered in anyway (Edmondson *et al.*, 1988).

Radial and tangential migration of the GCs relies on various indications originating from the nearby ground substances. The fate of some cells and the regulation of its migratory pattern in the EGL may be determined by intrinsic programs (Jankovski *et al.,* 1996). Granule cells initially go through radial migration across a collection of neurites, and later change their arrangement abruptly by 90° and then change the position of their nucleus in the newly formed neurite by not making contact with glia in microexplant cultures, (Nagata and Nakatsuji, 1990). Alterations in the path by which cell migrate is possibly controlled by inherent indicators and the organization of the framework of the cell (Rakic *et al.*, 1996).

Granule cells that migrate express numerous genetic factors at definite landmarks on their migratory route and few of these genes are programmed for the special organs that react to changes that are turned on by the cell adhesion molecules (CAM) (Hatten *et al.,* 1997). Signals emanating from the cells of the EGL in conjunction with interchanging expression of gene in tangential movement of GCs may act to adjust shape of the cells and pattern of migration.



EGL	External granular layer
ML	Molecular layer
P	Postnatal Day
dWM	Deep White matter

Figure 2.7: Changes in the developing cerebellar cortex in mice (Source: Komuro *et al.*, 2001).

It is established that many GCs move tangentially, at right angle to the alignment of the parasagittal compartments, this shows that cells are channeled to go through a particular section prior to the beginning of radial migration. Proper distribution of the species-specific quantity of GCs to each PC is coordinated by signs originating within their shorter branches (Zecevic and Rakic, 1978) and this might attract or repel. As the frontal part of the brain develops, young cells that are fated for the radial columns or cerebral cortical layers appear to be specific for proliferative activities in the VZ, prior to the commencement of radial migration (McConnell and Kaznowski, 1995). It is therefore a possibility that the GCs of the cerebellum also are fated to inhabit a specific anterior-posterior (A-P) compartment comparable to the pattern of formation in the growing forebrain. It remains uncertain whether postmitotic GCs move past the A-P compartment border in the course of their divergent transit in the EGL prior to inhabiting a particular A-P segment, seeing that each A-P segment differs in location, breadth, and from each other (Herrup and Kuemerle, 1997). Importantly, the radial migration of GCs takes place mostly in the classified ways (namely GC raphes) that connect the EGL with IGL (Lin and Cepko, 1998). The presence of GC raphes proposes that GCs that have divided may travel tangentially till they get to the site of GC raphes in the EGL. In the mice, GC raphes are yet to be identified. Responding to surrounding environmental factors, tangentially migrating GCs begin radial migration. Confocal microscopy allows an extensive investigation of the function of different receptors and components that are necessary for tangential migration in the normal physiological state (Komuro *et al.*, 2001).

2.5 CEREBELLAR CORTEX

Components have a distinct geometric arrangement, which is sorted in relation to the transverse, longitudinal and tangential planes in each folium. Within the cerebellar gray matter are located the terminations of mossy and afferent climbing fibres, five different neurons (basket, outer stellate, Purkinje, granular, and Golgi), neuroglia and vascular components.

Three discrete layers make up the CC: molecular, Purkinje and granular layers. The major cerebellar circuit includes the GCs, PCs and neuronal elements in the CN. The end portions of the mossy fibre afferents are received by axons of GCs which climb to the ML, and divide into parallel fibres. Purkinje cells are the largest and the lone cells that are sent outside of the cerebellar gray matter. Longer branches of GCs end in the vestibular and CN. The dendritic trees

of the PCs cells accept the end parts coming from the climbing fibres which are produced in the neurons found in the inferior olivary nucleus, alongside the dense arrangement of the parallel fibres. There are two types of input are received by the CC: olivocerebellar climbing fibres, which forms a communication with PCs and mossy fibres, whose axons are the parallel fibres which is associated with the PCs through GCs (Johna, 2008).

Within the cerebellum are found few types of cells that are located in the CC and CN. The neuronal components in the cerebellar cortex consists of outer SCs and BCs in the ML, PCs and candelabrum cells in the PCL, and GCs, UBCs, GoCs and Lugaro cells within the IGL. Positioned at the superior part of the 4^{th} ventricle in the cerebellar WM are the neurons of the CN. In the cerebellum, the main output consists of the CN and some vestibular nuclei.

Smallest amidst the neuronal types in the cerebellum are the GCs which outnumber all other classes of neurons found in the CNS, responsible for about 4/5 of the entirety of nerves in human and their precise growth establish and dictate the general development and histoarchitecture of the cerebellum (Altman and Bayer, 1997). In mice, abnormal formation or death of GCs results in very critical deformities in the cerebellum, which includes a number of disease conditions linked with granule cell aplasia common in human (ten Donkelaar *et al.*, 2003). Proper proliferation of granule neuron precursors extends till about the PND 15 in mice and one and a half years in humans. From granule cells rise parallel fibers which are classified glutamatergic.

Far-reaching research has revealed quantitative data about the CC- as having a total surface area of approximately 200,000 square millimeters containing:

about 500 Purkinje neurons, about 600 basket cells, about 50 Golgi neurons, about 3,000,000 granule cells, about 600,000 glomeruli (Inderbir, 2014). Each axon reaching the cerebellar cortex from the olive divides into about ten climbing fibres. Each olivary neuron, therefore, establishes connections with about ten Purkinje cells. Each mossy fibre synapses with about 400 granule cells. The axons of each granule cell synapse with about 300 to 450 Purkinje neurons. Each Purkinje neuron may bear about 80,000 synapses with separate parallel fibres. The arborisations of a lone Purkinje cell may be crossed by about 250,000 parallel fibres. The entirety of neurons in cerebellum far exceeds that of the remaining parts of the brain (Inderbir, 2014).

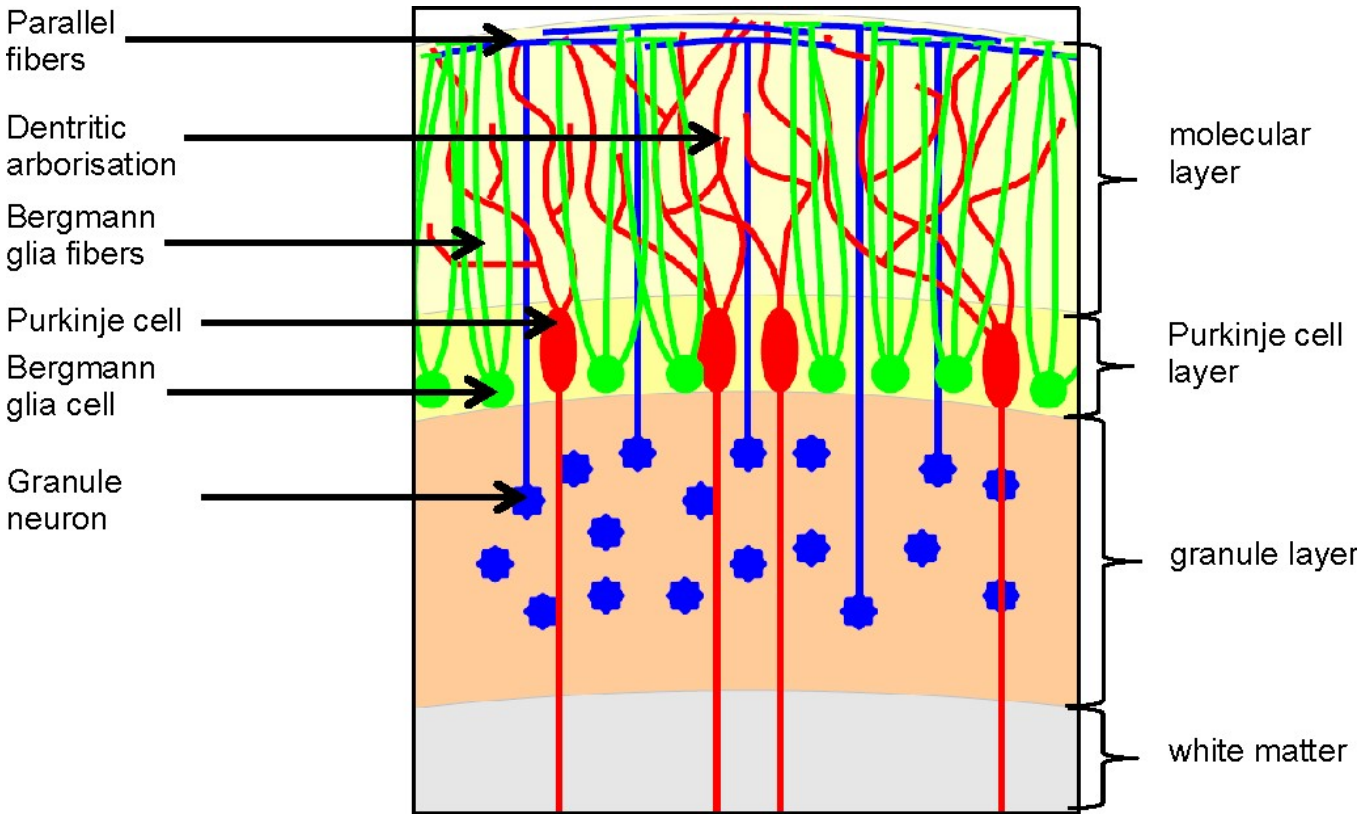
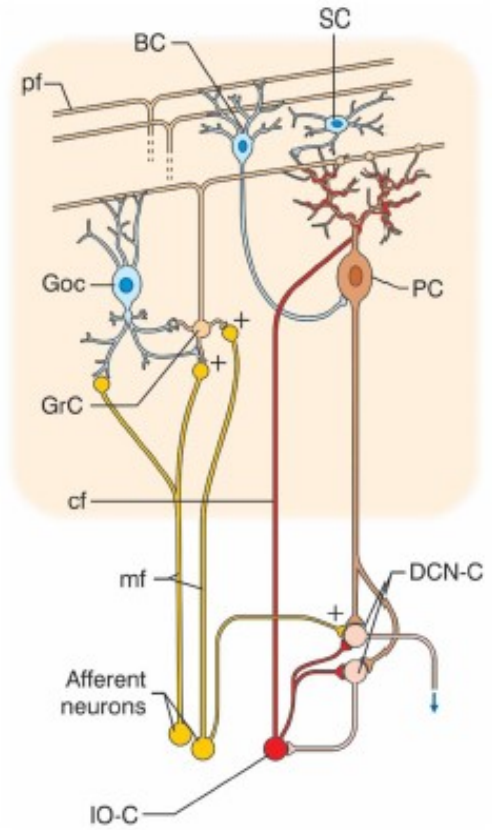


Figure 2.8: Different cell types and fibres found in the cerebellar cortex. (Source: Semanticsscholar.com)

2.6 SYNAPSES OF THE CEREBELLAR CORTICAL NEURONS

Cerebellar cortical neuron fibers form communication on all the diverse classes of nerve cells in the ML. The contacts with PCs are on spines while the synapses on the rest are on the dendritic shafts. It is controlled primarily by activating the sonic hedgehog signaling pathway (Wechsler-Reya *et al*., 2001). Other mechanisms which include Igf-2 or Sdf-1 signaling are also implicated in the multiplication of granule neuron precursors (Hartmann *et al*., 2005).

Within the IGL are small granular cell bodies which are arranged into few dendrites whose input are from afferent fibres in the cerebellum (Chédotal, 2010). Majority of the afferent fibres constituted in the cerebellum are mossy fibres in the adult cerebellum, they are seen projecting to the Purkinje cells (Voogd, 2011). Inferior olivary nucleus produces all the climbing fibers which also synapse on the Purkinje cell dendrites, both of these fibres are glutamatergic. For lamination of the cerebellar cortex to occur, granule neurons and Bergmann glia are indispensable (Sotelo, 2004; Wang and Zoghbi, 2001).



BC	Basket cell
SC	Stellate cell
Pf	Parallel fibres
PC	Purkinje cell
GoC	Golgi cell
GrC	Granule cell
DCN	Deep cerebellar nuclei
Cf	Climbing fibres
Mf	Mossy fibers

Figure 2.9: Schematic representation of cerebellar cortical circuit GoCs, SCs and BCs, PCs, GCs. (Source: D'Angelo and Casali, 2013).

2.7 LAYERS OF THE CEREBELLUM

2.7.1 Molecular layer

This is the first part of the CC, it lies underneath the pia mater and consists of superficially located long and short branches of SCs and BCs, cell bodies and dendrites of PCs. Within this layer are GC axons and glial cells. In humans, the ML is between 300mm - 400mm thick. It is sparsely populated with neurons, axons that are unmyelinated, dendritic arborizations and neuroglial radial fibres. Dendritic trees of PCs extend to the periphery and widen out at right angle to the cerebellar folia long axis. Purkinje cell dendrites are flat. Laterally, the dendritic size is more in the horizontal level than in a level corresponding to the cerebellar folia. Parallel fibre stems climb into the ML where they split into twigs looking like "T". Both branches spread in opposed routes as parallel fibres on a folium. The termination of parallel fibers is on the shorter branch of the PCs and GoCs, and also on the SCs and BCs of the ML. Golgi neuron dendrites extend outwardly to the exterior and cover the territory of many Purkinje neurons longitudinally and transversely. Nerve communication exists between Parallel fibres and these dendrites. Dendrites of some Golgi cells enter the IGL, and make contacts with mossy fibre terminals. Within the ML are found dendrites and axons of stellate neurons and their somata, which are superficially located in the ML while cell bodies of BCs lie inwardly in this region. Climbing fibres contact the Purkinje dendrites in the molecular layer as they ascend up through the granular layer. Bergmann glial cell processes have radiating branches which surround all neuronal elements, with the exception of their synapses. The conical expansions of Bergmann glia unite to create an outer limiting sheet on the outside of the cerebellum (Johna, 2008).

2.7.2 Purkinje cell layer

Within this layer are found the large, flask-shaped cell body of the PCs, seen only in the cerebellum. This layer is located amidst the ML and IGL in the cerebellar cortex. It is composed of a monolayer of PCs, GABA-ergic projection neurons known for their large somata. Present in this cell layer are the smaller somata of Bergmann glia, which are specialized glia that are different from Purkinje cells by their ramifications around the PCs which often radiate into the ML. Occasionally, Golgi cells infiltrate in between the Purkinje cell somata. Most PC axons leave the internal limit of the cell body and cross the IGL to infiltrate the WM which lies beneath. This cell layer is evident in every subdivision of the cerebellar cortex (Johna, 2008).

2.7.3 Granular layer

This intermediate layer is about 100µm wide in the fissures and 400-500µm on the summits located within the folia. Granule neurons account for about 2.7 million granule neurons per mm^3. The human cerebellum is projected to contain an estimate of 4.6 $x10^{10}$ granule cells and 3000 granule cells are allocated to each Purkinje cell (Johna, 2008). This portion is the deepest of the cerebellar cortex. It comprises of tiny GCs and glomeruli**.** Granule cells are classified as being among the smallest cells in the body with an estimated diameter between 4-5µm. Cells in this layer are packed densely together in contrast to the cells of the ML which are less dense.

Granular layer contains the cell bodies of GCs, the first part of their long branches; short branches of GCs; subdivisions of the end parts of the longer branches of afferent mossy fibers; climbing fibers ascending from one end to the other end of the GL before reaching the ML; and the soma, dendrites at the bases and intricate axonal network of GoCs. Cerebellar glomerulli are synaptic rosettes made up of a mossy fiber endpiece which establishes synapses that are stimulated on the short branches of GoCs with GCs (Johna, 2008).

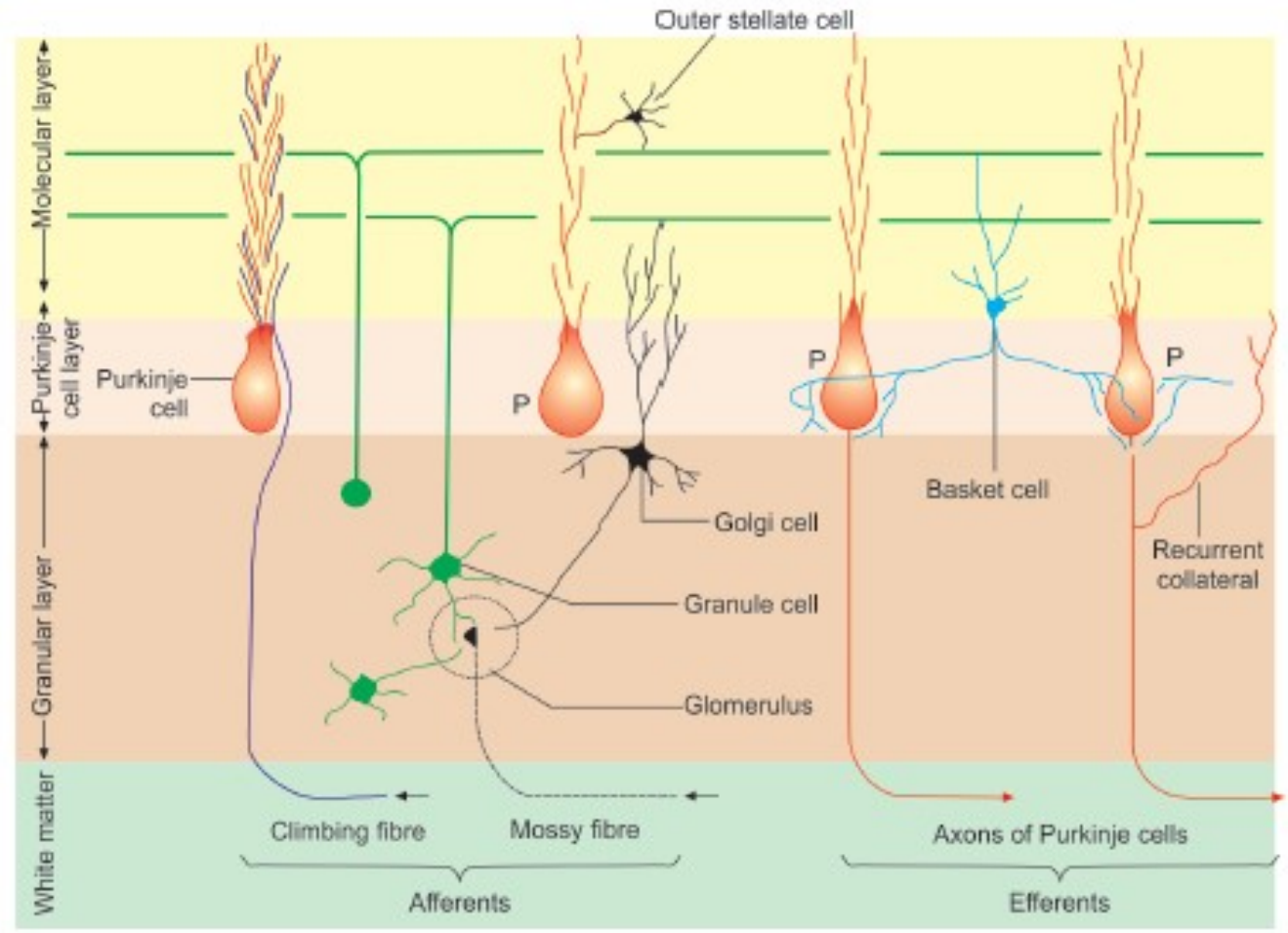


Figure 2.10: Scheme to show the arrangement of neurons in cerebellar cortex (Source: Inderbir, 2014).

2.8 CELLS OF THE CEREBELLUM

2.8.1 Granule Cells (GCs)

Term used to refer to neurons characterized by small cell bodies. These are more numerous than other neurons in the mammalian CNS. Granule cells are found in various regions of the brain including the cerebellum, cerebral cortex, hippocampus and olfactory bulb. These cells perform very vital function in the activities involving mossy fiber input into the cerebellum. The GCs are small neurons with a perikaryon between 7 to 10 μm in diameter. These cells outline a different layer amidst the PCL and WM in the cerebellum. A vast number of neurons in this part are GCs which inlude not too many glial cells and SCs mingled between them. The cells possess a big nucleus and a slim circumference filled with cellular fluid. Shorter branches of these nerve cells are rather tiny and little and may not be traceable from the soma in brain slices that are thin. Dendritic canaliculi are observed in the shorter branches that are nearer while the long branches pass upward to the ML of the cerebellum in light microscopic sections. These axons are not myelinated and are complex to mark out in sections that are thin. Granule cells divide and run horizontally in the molecular layer (Fox, 1962), and as a result they form plenty parallel fibers in electron micrographs of the ML.

In properly prepared sections, genetic materials are spreaded equally in the nucleus without a lucid area. In poorly fixed specimens, there is clumping of chromatin at the edge of the nucleus with lucid portions in the middle. A notch in the nucleus is often observed commonly positioned near the spot where the cellular fluids are enlarged to lodge the Golgi apparatus, few granular endoplasmic reticulum, a multivesicular body and few dense bodies. The cytoplasm contains a thin brim consisting of sheets of the endoplasmic reticulum and few mitochondria. Golgi apparatus is made up of membranes without granules with vesicles similar to those seen in other neurons, although it is not as broad as those in large nerve cells. The dense bodies are different from those present in the PC because they are quite tiny and generally enclose numerously thick particles.

The GC is the chief cell in the cerebellar gray matter, making up as much as fifty percent of all neuronal cell types in the CNS. These cells come from a germinative region in the uRL (Alder *et al.*, 1996). On the 13^{th} day of embryogenesis, GCPs egress the RL and move in an upper manner across the border of the cerebellar anlage to constitute an area known for multiple cell divisions called the EGL, which is formed on the 15^{th} embryonic day, and GCPs within the EGL maintain

the power to constantly divide for 21 days after delivery (Miale and Sidman, 1961). At parturition, some GCPs begin to leave the cell cycle to become fully developed GCs which results in the steady dissolution of the EGL. Initially, GCs send the longer branches of nerves to the pia and later travel from the centre on BG to settle in the IGL, the ultimate location of GCs (Adams *et al*., 2002). The IGL can be seen on PND 5 and is accomplished by PND 20 (Altman and Bayer, 1997).

In the hippocampus and cerebellum, GCs are known for a remarkably delayed maturation (Smart, 1982). In both brain areas, these neurons maturate in the third trimester of gestation in man (Dobbing and Sands, 1973), equivalent to the initial period after birth in mice and rats from the 10^{th} embryonic day to PND 20 (Reznikov, 1991).This time frame is exposed to different insults that may end in permanent brain damage.

2.8.2 Stellate Neurons

"Stellate neuron" collectively refers to all the neurons found in the CC excluding GCs and PCs (Herndon, 1964). The somata of these neurons range from 6 - 20µ in diameter.

Throughout the molecular layer are tiny stellate neurons dispersed. The shape of their nucleus is round or oval which is encapsulated by a two-layered membrane revealing some nuclear pores. An infinitessimal nucleolus is frequently present in the nucleus. Within the cytoplasm which has a thin rim is located the Golgi apparatus and components of the granular endoplasmic reticulum are spotted. Other organelles seen include a number of mitochondria and a few dense bodies. These cells possess few processes which are difficult to trace. In the proximal cell processes dendritic canaliculi may be seen.

In stellate neurons that are large, nuclei have a complex two-layered nucleolemma and a conspicuous nucleolus. The cellular component excluding the nuleus is large and consists of a huge Golgi apparatus, mitochondria, endoplasmic reticulum, dense bodies and a number of large dendrites with abundant dendritic canaliculi.

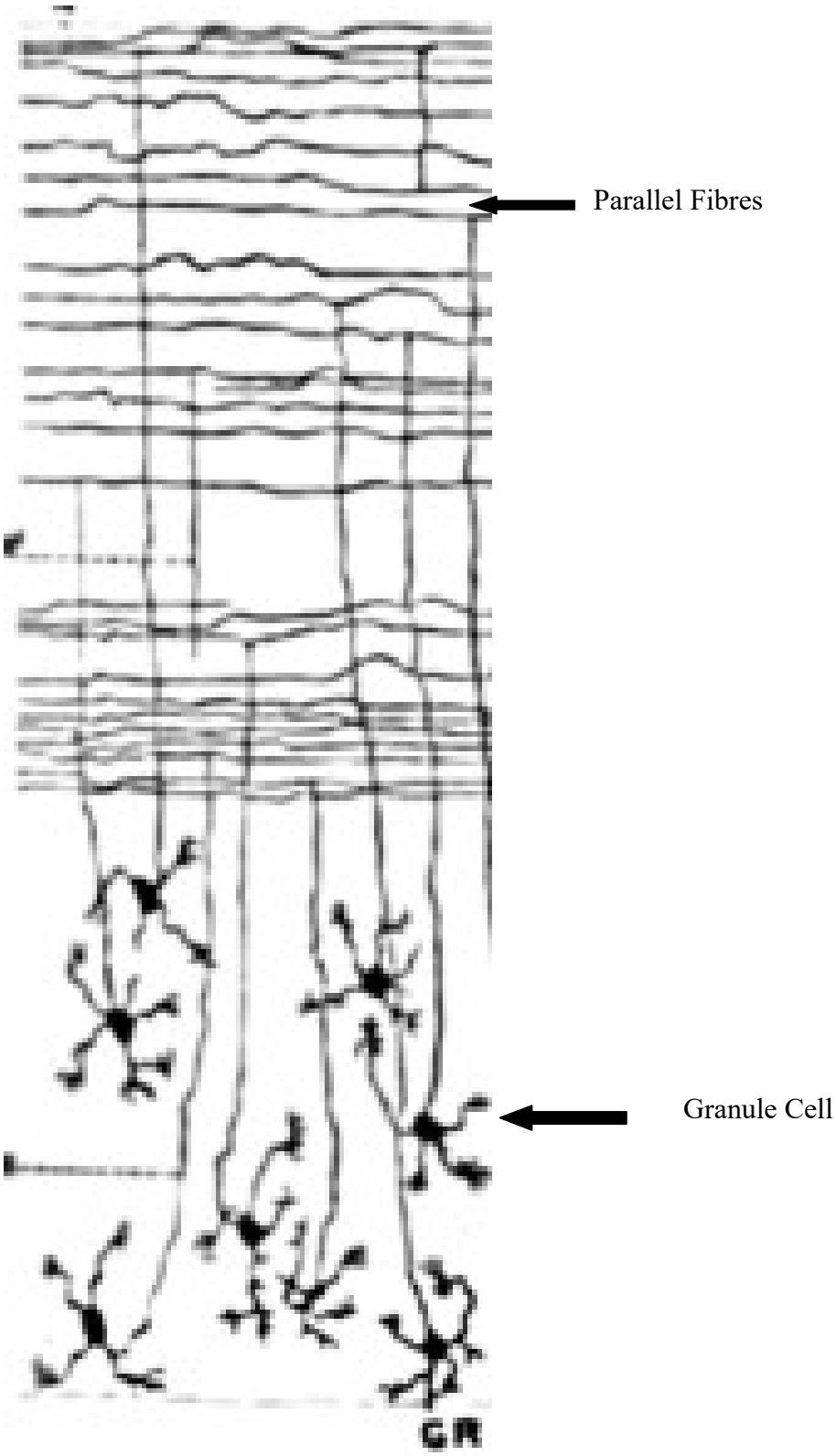


Figure 2.11: Granule cell showing soma, parallel fibres and basal dendrites (Source: Linas *et al*.,

2.8.3 Purkinje Cells

Large neurons in the mammalian braininclude Purkinjecells (Purves *et al.*, 2008). These cells have a delicately highly structured dendritic arbor, which is distinguished by a vast array of dendritic spines. PCs are seen in the PCL in the cerebellar cortex. These neurons have enormous dendritic arbors which form an easy passageway through which parallel fibers go by. Parallel fibers provide fragile excitatory connections between nerve cells to spines in the shorter branch of the PC, while climbing fibers whose origin is within the inferior olivary nucleus in the medulla confer strong stimulatory input to the first shorter branch and somata. Arbor of Purkinje neuron dendrites measures up to 200,000 parallel fibers which allow these fibers to pass through (Tyrrell and Willshaw, 1992) and form a granule-cell-Purkinje-cell synapse with each PC. Every PC gets about 500 climbing fiber synapses which are derived from one climbing fiber (Wadiche and Jahr, 2001). Basket and stellate cells both inhibit the PCs, while BCs make connections on the first part of the PC axon, SCs form a link with the dendrites.

Purkinje cells make up the major output of all motor coordination cells and they launch inhibitory projections to the DCN in the CC.

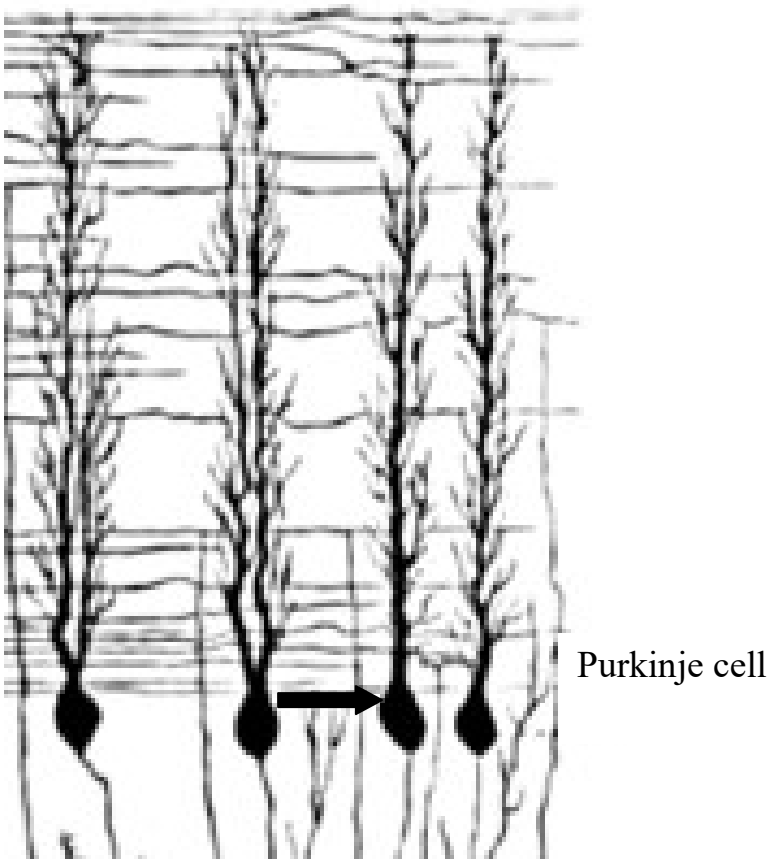


Figure 2.12: Purkinje cells showing flattened dendritic tree (Linas *et al*., 2004).

2.8.4 Unipolar Brush Cells (UBCs)

These cells are a group of connecting nerve cells that are small and excitatory related with cerebellar gray matter and structures that are similar to the cerebellum (Mugnaini *et al*., 2011). They are found in large numbers in the flocculonodular lobe (Sekerková et al., 2014) and the DCN (Borges-Merjane *et al*., 2015). Knowledge of unipolar brush cells (UBCs) derives solely from researches done in laboratory animals, the major attributes of UBCs are greatly preserved among the different classes of animals : these cells are documented in teleosts and are located in all mammals, humans inclusive (Bazwinsky *et al*., 2008).

A characteristic UBC possess a dendrite that is thick which terminates in a network of tiny dendrioles, which produces a special synaptic junction in the company of the ends of each mossy fiber (Diño *et al*., 2000). Axons of UBC divide within the granular layer and generate a built-in mossy fiber system overlaid on the one that is external (Berthié *et al*., 1994; Nunzi and Mugnaini, 2000). These features characterize all UBCs and are grouped into category I and category II, indicating the two functionally and chemically discrete subcategories (van Dorp *et al.*, 2014).

In rats, type I UBCs are produced between the 16th -19^{th} embryonic day, 14^{th} – 17^{th} embryonic day in mouse while type II UBCs are formed for an extended time frame 18^{th} embryonic day (in mouse, 16^{th} day of embryogenesis) to a few hours after birth PND 0–1. They were previously assumed to have been derived from the EGL (Abbott and Jacobowitz, 1995), recently, histological studies reveal a VZ origin (Ilijic *et al*., 2005).

A good number UBCs arrive at the granular layer by PND 10, some days preceeding the end of GC neurogenesis. The terminal transformation of UBCs takes place from PND 2 and ends on PND 28. The development of type I UBCs has been grouped into four specific parts depending on the gross outlook of the dendritic brush into: filopodial, protodendritic, dendriolar brush and transitional brush stages. In the final phase (PND 21 – PND 28), the UBCs start to exhibit mature UBC morphology (Morin *et al*., 2001).

2.8.5 Astrocytes

Cerebellar astrocytes have similar cellular features excluding the Bergmann astrocytes (Golgi epithelial cells) being a separate category. Other astrocytes have a nucleus that is either oval or round. Nuclear chromatin is equally spread, contains more granules and it is less dense than GCs

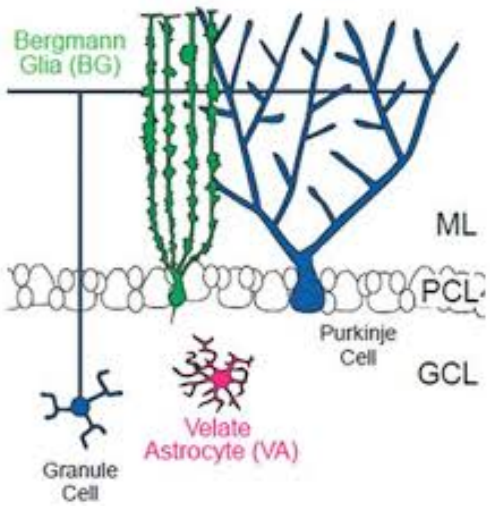
or oligodendrocytes. Present in the nucleus is a small nucleolus. The cytoplasm of astrocytes is less thick than that what obtains in other cells located in the cerebellum; granular endoplasmic reticulum consists of fewer scattered elements, dense bodies differing in number, size, and shape between cells which are frequently wrapped inside a membrane and contain delicate granular dense bodies. Within the cytoplasm are glial fibrils which run in bundles and into the various processes of a cell. In the IGL, branches of the astrocytes are smaller and contain fewer glia fibrils than branches in the astrocytes in the WM.

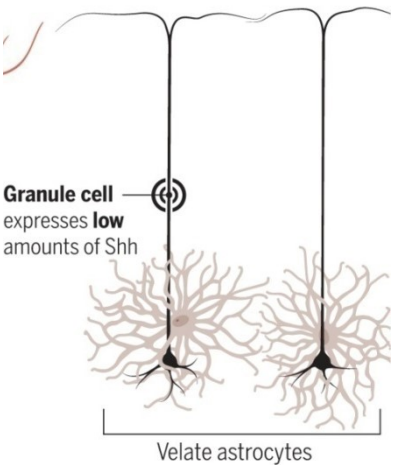
2.8.6 Bergmann Astrocytes

Another name for these cells is unipolar cerebellar astrocytes present in the VZ, they are formed from radial glial (RG) cells. In the later weeks of embryogenesis, these glia move to the cerebellum in to the PCL, extending radial fibers to the periphery of the pia shortly after delivery (Chanas-Sacre *et al.*, 2000). Radial fibers of BG cells give direction to migrating GCs and establish synapses with shorter branches of PCs (Yamada and Watanabe, 2002). Granule cells use BG as scaffold for transiting from the EGL to the IGL.

The cytoplasm is less dense than other astrocytes. The Golgi apparatus is apparently not large. The rough endoplasmic reticulum is not well formed and looks like a tube in shape. Multivesicular bodies may be observed. The quantity of dense bodies is not constant; they are found grouped together near the nucleus or at the inferior part of the branches. The mitochondria are more dense than in several categories and it is not easy to identify their cristae.

Bergmann glia are quick to be recognized because due to their orientation and morphology within the cerebellar cortex. The following features are used to identify cells aesthetically as BG: (1) 3–6 thick radial bodies reaching the pia from the ML, (2) minute soma in the PCL, (3) end-feet that reach the pia, (4) tiny protuberances spreading from the key shafts of glia (Grosche *et al*., 1999), and (5) absence of an axon.





ML	Molecular layer
PCL	Purkinje cell layer
GCL	Granule cell layer

Figure 2.13: Velate astrocytes showing their numerous processes (Source: alz forum.org and sciencemag.org)

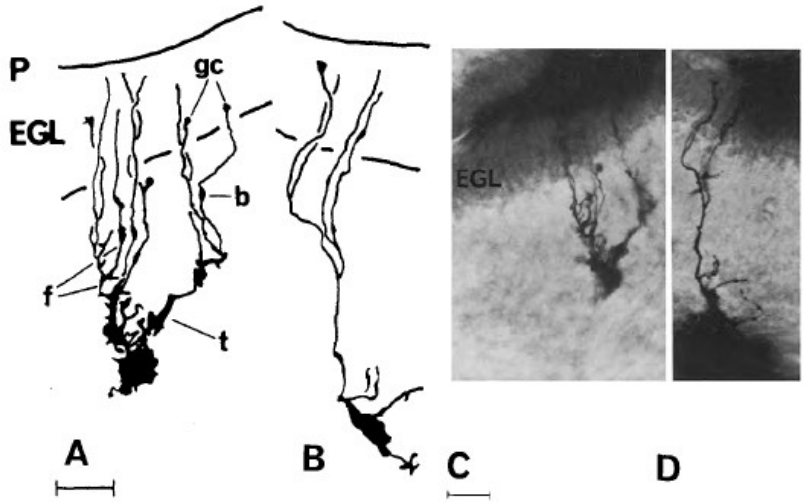


Figure 2.14: Bergmann glia cells in the vermis of a PND 2 rat

t- thick fibres EGL- External granular layer

f- filiform fibres

b- beady enlargements

gc- growth cones (Source: Shiga *et al.,* 1983).

Surface area of each BG expands almost 50-fold in the initial 3 weeks after delivery in a mouse (Grosche *et al*., 2002), following which many more excitatory synapses in the cerebellar gray matter are enveloped by BG cell.

The hypothesis of contact guidance described by Rakic in 1971 is established to a certain extent in man (Choi and Lapham, 1980) and monkey (Levitt and Rakic, 1980). When development begins, BG cells support the movement of GCs. The source of BG is not exactly clear. Early experiments in mouse (Sommer *et al*., 1981), apes, and man proposed they are the altered radial glia which arise from the VZ. Alternatively, the BGs located in the rat cerebellum are derived from the EGL and commence their movement downwards on PND 6 as revealed by Golgi-Cox study (Das, 1976). Also, using autoradiography, mouse cerebellum showed that few glia may be formed in the EGL (Fujita 1967). Golgi study indicated that GCs were previously located between PCs on PND 0 and PND 2, and Bergmann glial cell filiform fiber penetrate the EGL on PND 0. There was a gradual increase in the quantity of the fibers, alongside the maturation of the EGL and this reaches a maximum on PND 8. Afterwards, the quantity of fibers alongside width of the EGL diminished (Shiga *et al*., 1983).

The EGL cleared just before PND 25 (Altman, 1969) and a BG has about 4 fibers on PND 25. The simultaneous changes in the EGL thickness and quantity of BG fiber proposed a close relationship amidst both. Beside the whole stretch of the EGL, this portion of the cerebellum also increases in the first two weeks after birth (Heinsen, 1977), this results in a boost in the capacity of the EGL and, consequently, an increase in the total EGL cells while many more BG persisted a proliferation for the next 14 days (Shiga *et al*., 1983). A likely role as substrate for directing more granule cells that migrate is the proliferation of the BG fibers which arise from the expansion of BG cells and the number of fibers in each cell. Before PND 8, not many thick spreading fibers were seen on the exterior of the filiform fibers. On PND 12, many thick expansions showed up and their quantity rose slowly. On PND 15, a number of fibers were overlaid with expansions throughout. Rakic (1971) reported that the GCs travel on the BG fibers, by evading the expansions on the periphery by moving in the region of the glia body. The movement of GCs on the BG fibers with surface covered mostly with thick expansions is not clear.

The major types of cerebellar cells comprise BG which are obtained from RG in the VZ of the 4^{th} ventricle except GCs which arise from the uRL. During the transformation between radial to

Bergmann glia which takes place between 14.5th and 18.5th day of embryogenesis in the mouse, Bergmann glia retain the radial glia processes at the base and move their cell body from the VZ to the potential PCL (Yuasa, 1996). Shortly after delivery, the BG radial fibers assist in the internal movement of maturing GCP starting at the EGL to produce the IGL (Xu *et al*., 2013). In the mature cerebellum, Bergmann glia retain configurational integrity and synaptic links (Yamada and Watanabe, 2002), and these are anticipated to be cells that are neurogenic (Koirala and Corfas, 2010).

2.8.7 Oligodendrocytes

These cells are a little smaller than granule cells or astrocytes. They are very abundant at the junction of the IGL and the WM, particularly beside Purkinje cells. Their nucleus is somewhat oval; genetic materials are distributed equally but it is possible that they clump to some extent at the margin.

The cytoplasm is made up of a thin edge bulged at one part at the location of Golgi apparatus. Arrayed around the nucleus is the endoplasmic reticulum. The mitochondrium is bigger than the surrounding nerve cells (Palay *et al.*, 1962), it has a thick matrix and cristae that are not well developed as a result of its huge density. The density of the cytoplasm is mostly owed to the presence of many liberated RNP particles.

2.8.8 Microglia

These nerve cells are scarcely distributed in the cerebellum, although they are commoner in older animals. These cells have very dense cytoplasm with elongated nuclei. The nucleus is thick especially at the margin; centrally, the nucleoplasm might contain more granules with some regions less thick amidst the granules. It has abundant cytoplasm within which are rudiments of the granular ER and RNP particles. The Golgi apparatus is big and conspicuous within the dense cytoplasm. There are fewer mitochondria which are lesser in size compared to those in other nerve cells. Few dense bodies might be available.

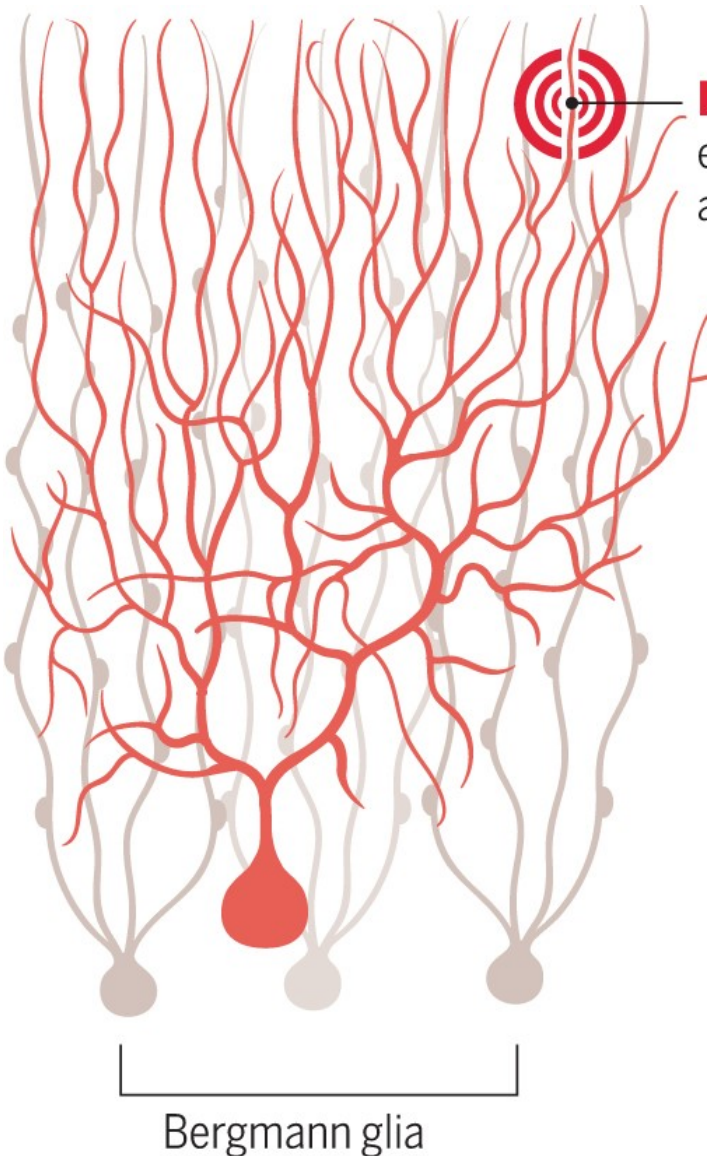


Figure 2.15: Bergmann glia showing its ramification around Purkinje cell soma (Sciencemag.org)

2.8.9 Lugaro Neurons

These cells are usually found superficially in the IGL beneath the PC bodies. Some Lugaro neurons are found amidst PC and IGL and these possess fusiform somata; others are found deeper in the IGL and possess triangular cell bodies. They are large cells considering their soma size. They have a typical number of 3.9 primary dendrites in each neuron with spindle-shaped somata that are horizontally arranged in the parasagittal plane. Dendrites are not too branched and extend over 100μm. Cells with triangularly shaped cell bodies face different directions, some may even lie perpendicular to the PCL. Contrary to the other cerebellar neurons which are fairly consistent histologically, Lugaro neurons vary in species (Jacobs *et al.*, 2014).

2.9 GLIOGENESIS IN THE CEREBELLUM

Within the cerebellum, the production of glia matches the production of granule cells and other interneurons. This is not the same as the other CNS regions where neurogenesis comes before gliogenesis. The adult cerebellum of mammals has four categories of astroglial subtypes, this includes stellate multipolar astrocytes, protoplasmic astrocytes within the IGL, fibrous astrocytes in the WM and the neuroepithelial cells showing radial Bergmann branches at the bases extending from the somata in the PCL throughout the whole ML and to the basement membrane underneath the pia (Altman and Bayer, 1997).

Using comparative analysis in various mammals, it was reported that cerebellar astroglia are from the VZ and that RG parent cells at the VZ were aimed at depending on the manner in which stem-cell markers were expressed (Yuasa, 1996; Anthony and Heintz, 2008), or VZ-restricted tags. Rhombic lip also contributes to cerebellar astrogliogenesis as proposed by (Jensen *et al*., 2004) but this remains unclear. Anatomical examinations and exploration of these pathways showed an initial astrogliogenic signal (up to about 14th day of embryogenesis in mouse), where radial glia are removed from the ventricle and displaced towards the nascent prospective white matter (PWM), where they change into the precursors of Bergmann glia (Yamada and Watanabe, 2002; Li *et al*., 2014). Some of these precursors seems to have undergone mitosis already when translocation is about to take place (Sudarov *et al.*, 2011) and transforms readily into Bergmann glia. On the other hand, other progenitors form a proliferative layer that continues to grow (Yamada and Watanabe, 2002) till PND 7 in consolidation with the tangential growth of the cerebellar area. Further phases of embryonic maturation include a second signal of astroglial-like

progenitors that do not have basal processes and these delaminate from the VZ into the PWM of the cerebellum where proliferation takes place, becoming astrocytes that will populate the prospective IGL and WM (Yamada and Watanabe, 2002). Bergmann glia morphogenesis needs a highly controlled contact with the nearby microenvironment of the cerebellum which includes basement membrane, Purkinje cells, GCs. Injury to these processes leads to the wrong placement of Bergmann glia and/or the possession of a star-like appearance, which may be a faulty maturation route for cerebellar astroglial progenitors.

Contrary to what obtains in the astrocytes, no proof clearly indicates the production of oligodendrocytes in the cerebellar VZ (Grimaldi *et al*., 2009). Instead, a small fraction of oligodendroglia appears to be derived from precursors in subventricular positions, most likely located in the PWM (Sudarov *et al.*, 2011). Conversely, transplantation experiments in the mice point toward an origin other than the cerebellum for a vast number of oligodendrocytes (Grimaldi *et al*., 2009).

As soon as the oligodendrocytes precursors finally settle in the cerebellar primordium, they initially enclose the CN and slowly occupy the newly formed cortical lobules, moving gradually in a centrifugal way. A similar blueprint is seen in the manner of differentiation and myelination that begins from the deep regions of the cerebellum to the apex of the lobules (Rossi *et al*., 2006). Majority of intrinsic and extrinsic processes that control oligodendrocyte differentiation are in various CNS regions, the cerebellum inclusive. Regulation and development of oligodendroglia in the cerebellum is controlled by hormones secreted by the thyroid gland and Purkinje cell - derived signals. Purkinje cells secrete sonic hedgehog shh, this enhances oligodendrocyte progenitor proliferation at the initial stages after birth, while by the end of PND 7, the progenitors start secreting vitronectin, which stimulates the growth of oligodendrocyte and the production of myelin (Bouslama-Oueghlani *et al.*, 2012).

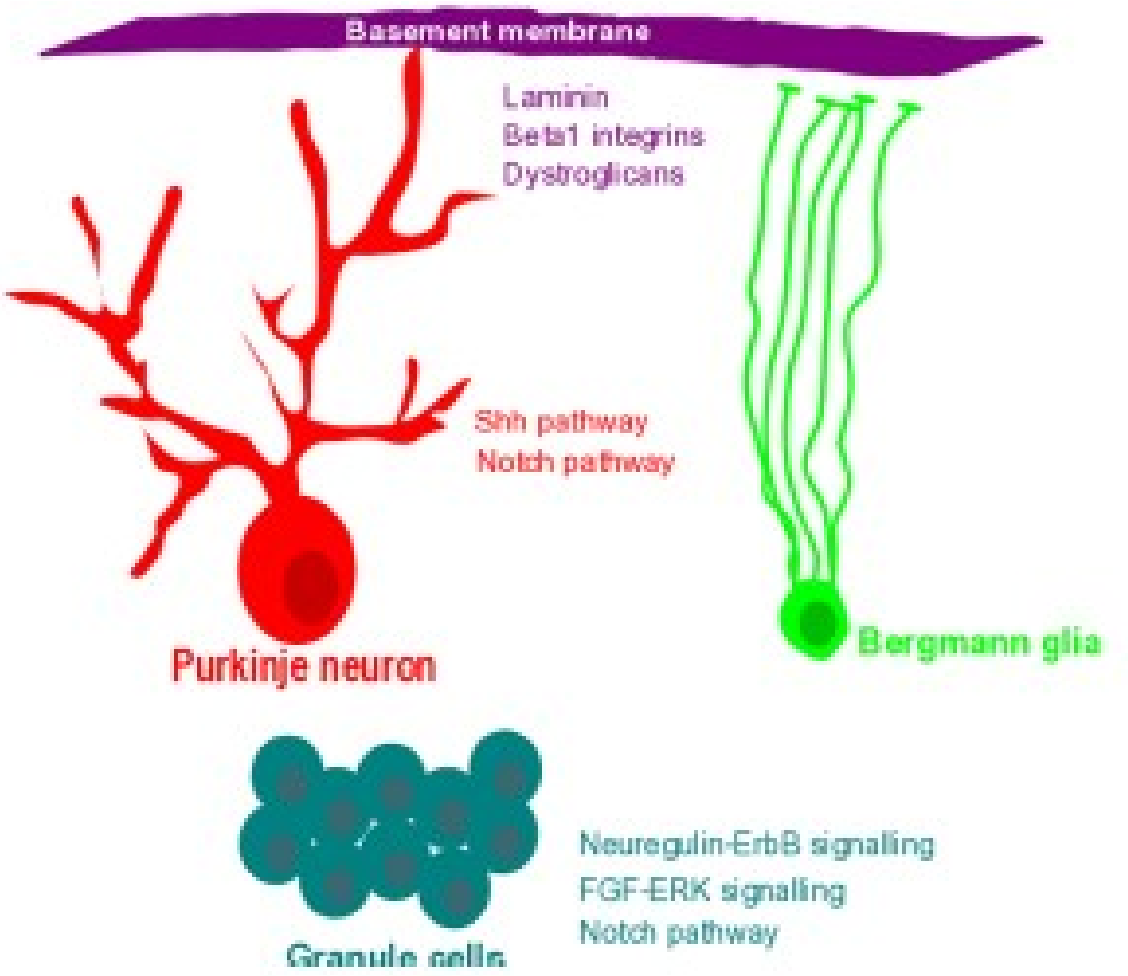


Figure 2.16 Components of the cerebellar microenvironment (Source: Leto *et al*., 2015).

2.10 ROLE OF SONIC HEDGEHOG (Shh) IN THE DEVELOPMENT OF CEREBELLUM

The activity of Shh in the cerebellum has been studied at length in granule cell precursor proliferation. Recent studies showed more function for this pathway at various levels of the growth of the cerebellum. Sonic hedgehog is a principal factor that necessitates the division of neurons for the development of diverse kinds of glial and neuronal cells from all sorts of precursors. The manner by which Shh enhances increase in these precursors seems to stand out, as this involves both cerebellar-like and other methods. In the cerebellum, secretion of Shh is limited to PCs beginning at embryonic day 16.5 and continues to adulthood (Huang *et al*., 2010). The initial segment of Shh signaling is vital for a quick clonal expansion of granule cell precursors as blocking Shh signaling in Purkinje cells diminshed the quantity of GCPs in the EGL.

Besides GCPs, shh expression is needed in support of the growth of GABAergic interneurons by controlling the precursors in two differing regions of neurogenesis (Fleming *et al*., 2013). In the VZ niche, after E12.5 shh expression is made active in multipotent radial glial cells. Malfunctioning of Shh expression in VZ drastically damages the increase in the quantity of RG and the power to produce GABAergic interneuron parent cells in utero (Huang *et al*., 2010). Alternatively, continuous use of Shh signaling amplified the quantity greatly. The origin of Shh expression that act on VZ radial glial cells may be other than the cerebellum seeing that Shh signaling is yet to be seen in the developing Purkinje cell mass. Shh is profusely produced in the epithelium of the choroid plexus in the hindbrain. This substance is available in the CSF in embryos and is deposited at the adjoining VZ through a transventricular mechanism. The cerebellar VZ is also an origin for astroglia that are stem-like of the second category of regions of neurogenesis located in the PWM occuring in the period of delayed gestation and delivery (Fleming *et al*., 2013).

2.11 EXTERNAL GRANULAR LAYER

Active multiplication of neurons occurs in the EGL of the cerebellum of many animals after delivery, neurons such as outer SCs, BCs, and GCs are produced here (Fujita, 1967). This layer responds to x-ray irradiation, special viral infections and some radiomimetic chemicals

(Herdon*et al.*, 1971). Researchers have stated that damage to the EGL results in a number of attending anomalous cytoarchitecture of the cerebellum (Hopewell, 1974).

Neurons of the CNS are formed in the VZ by multipotent progenitor cells (Cepko *et al*., 1997). Parent cells of EGL of the cerebellum identified to generate only cerebellar GCs were thought to be a prominent exception to this rule. Cells of the EGL are produced in the apical part of the uRL which is the border of the posterior part of the brain enclosing the dorsal aperture to the 4th ventricle, and are located on the exterior of the maturing cerebellum (Cajal, 1911).

The EGL is an important model for studying neuronal development and is a major prospective origin of most brain malignancies in young persons (Kool *et al.*, 2008). Abundance of cells in the EGL is regulated by Shh generated by the PCs (Wechsler-Reya and Scott, 1999).

Stem cells undergo mitosis in the EGL and the cells move pass the ML beyond the PCL into the IGL. Throughout the development that occurs after delivery in the rat cerebellum, the EGL has a matrix region where two different subregions are distinctly distinguished: the proliferative and the premigratory zones (Altman, 1972). Cells that just divided in the EGLmove to the definitive destination prepared for them in the IGL (Rakic, 1971); the proper location of these cells is fundamental for the regular arrangement of cells and the patterning of synapses in regions of the brain that are laminated as seen in cerebellum (Rakic *et al*., 1994).

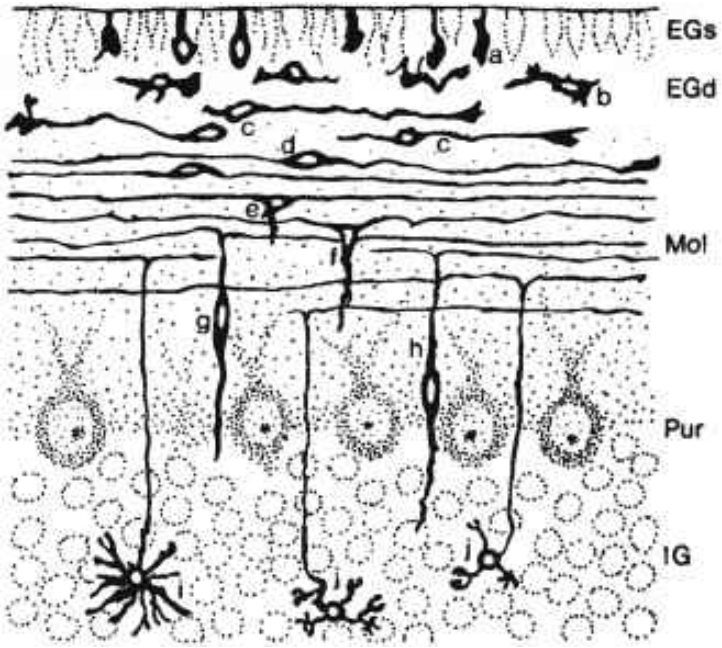
Apoptosis is needful for the appropriate expansion of the CNS. This thoroughly deletes cells in the proliferative part and the neurons that have undergone cell division (Blaschke *et al.*, 1998). A small quantity of degenerating pyknotic cells are seen at all levels of growth of the EGL within the cerebellum (Miale and Sidman, 1961). Between PND 5–9 GCs submit to a massive nuclear DNA fragmentation and it was inferred that cells that die came before synaptogenesis and may control the cell quantity (Wood *et al*., 1993). The uniqueness of the EGL among various populations of the CNS that proliferate is that it is next to the pia rather than on the exterior of the ventricle.

The EGL is a temporal layer which adds substantially to the quantity of neurons in the CC and regresses as soon as the cerebellum attains its full capacity.

The EGL is found in every vertebrate that has been studied as one layer of cells that has 6-8 cell diameter thickness. Cells in mitosis are located in all the outer region of this layer as an indication that interkinetic nuclear migration is not present, thus bringing similarities between the EGL and the SVZ. Proliferation of cells does not take occur in the interiormost portion of the

EGL, it contains cells that are preparing to migrate. Cells that comprise the IGL (majorly granule cells), are the principal output of this layer and represent the most plenteous neurons in the CNS. The cells in the EGL have a longer life span than the VZ from where the PCs of the cerebellum are derived. Purkinje cells develop within 72 hours from embryonic day 10 through 13 while the IGL cells are formed after a much longer period starting at the late gestational stage through the initial 21 days after delivery in the mouse (Miale and Sidman, 1961). The proliferation of the EGL from the uRL occurs at E17 in an anterodorsal direction. The long time it takes to produce neurons in the EGL is about the same in all animals (Zecevic and Rakic, 1976).

The production of PCs and GCs takes place in two different parts where there is vigorous multiplication of cells: the VZ of the 4th ventricle and the EGL at various periods as the embryo develops. It is now well understood why a typical cerebellar cortex with an adequate quantity of the two cell types will need a huge system that will regulate it and which will be required to provide a response by which the production of the granule cell will be influenced by Purkinje cell.



EGs	External granular (mitotic layer)
EGd	External granular (migratory layer)
Mol	Molecular layer
Pur	Purkinje cell
IG	Internal Granular layer

Figure 2.17 Cerebellar cortex showing the EG lying under the pia of a young cerebellum and other layers of the cerebellum (Jacobson *et al*., 1991).

Most degenerating neurons in the EGL are found in the post-mitotic region consisting of abnormally clumped heterochromatin indicating nuclear cell death (Smeyne and Goldowitz, 1989). A number of molecular and spatial relationships amidst neurons that migrate and the processes of the radial glial cells regulate this process (Ryder & Cepko, 1994). The quantity of GCs formed in the EGL is large, there may be as much as 2.2 $x10^9$ GCs present in the adult cerebellum, (Palkovitis, Magyar & Szentagothai, 1971). On PND 0, the EGL is made up of 3-4 cell layer and contains few structures that have undergone cell division throughout the whole length of the EGL subpialy to the newly formed ML. On PND 2, growth in the EGL has reached 4-6 cell layer, followed by an expansion in the quantity of nerves present. The mitotic index in the EGL is under 2%. At the boundary of the ML, there is a change in the outline of the GCs from round to fusiform. This alteration in cell morphology is steady with modifications seen in cells that migrate (Trinkhaus, 1984). Between PND 4 – 8, there is an increase in cell thickness of about 8-10 cell thickness and can be differentiated into two zones: an external zone (approximately 1/3 of the EGL) that consists mainly of mitotically sound, round cells, and an inner portion (about 2/3 of the EGL) that contains both round and lengthened cells that are waiting to migrate and it is not a very mitotically active zone.

2.12 DEVELOPMENT OF THE EXTERNAL GRANULAR LAYER

Central nervous system development follows the same mechanism of proliferation within a specified region seconded by the movement of the cells and transformation of these cells into mature types. Progenitors proliferate in the VZ in the cerebral cortex and these cells later form a discrete collection of discrete types of cells in the cortical layers. This process is modified in the CC (Altman and Bayer, 1997). Production of CGPs takes place in the uRL, where these cells form a second zone where proliferation occurs - the EGL and later move to the cerebellar primordium. Committed GCPs in the external region of the EGL multiply and leave the events that lead to the division of cells and to also change into other forms and move along the ML beyond the PCs to their final location when development after delivery proceeds. Cerebellar GCPs are formed in the uRL of the growing cerebellar primordium by progenitors which proliferate and move tangentially transversely on the cerebellar primordium to create the EGL in much later stages of development and maturation following delivery. It is not clear as to how the

precursor remains in the neonate or maybe GCPs are indicated wholly in the embryonic uRL (Silbereis *et al*., 2010).

A considerable migration occurs inward, whereby the cells of the EGL differentiate into the IGL cells and mingle with PCs and GoCs which are derived in the neuroepithelial layer amid PND 3 and 6. Immediately after hatching, the EGL disappears after it has finished its function in cell production (Hanaway, 2004).

Just after delivery, many pathways that are mitogenic increase the EGL from one row of cells to between six to eight cells thick. The influence of sonic hedgehog provided by PCs is a principal driver in GCP proliferation. The significance of shh to growth of cerebellar cells is highlighted by elaborate genetic examination showing that shh expression regulate the pattern of foliation in the CC (Corrales *et al.*, 2006).

2.13 MOVEMENT OF GRANULE CELLS FROM THE EXTERNAL INTO THE INTERNAL GRANULAR LAYER

In young CNS, cells are formed in specialized germinative zones and locate their final position through active migration. The process of migration is assumed to be controlled partly by genes and gene-like pointers which are perceived by the migrating cells.

A major part of the cerebellar cell growth include migration of neurons which occurs after birth making these processes easily open to experimental maneuvring in rodents, (Miale and Sidman, 1961). The movement of newly formed granule cells that have undergone mitosis from the cerebellar cortex has been well studied seeing it demonstrates a universal opinion in neuron-glia relationship, whereby neurons that migrate are steered by the radial branches of glia (Hatten *et al*., 1984).

All through the early period of proliferation, all external granule cells (EGCs) are engaged in mitosis and DNA synthesis. Path of migration and time of production of immature granule cell neurons have been studied (Altman, 1982). Shortly, following the final mitosis, GCs travel to a postmitotic zone where they remain for 21-24 hours and then begin migration into inner regions of cerebellar cortex. The migration of one series of EGCs from the EGL into the IGL takes an average of 35 to 45 hours in the mouse (Fujita, 1967).

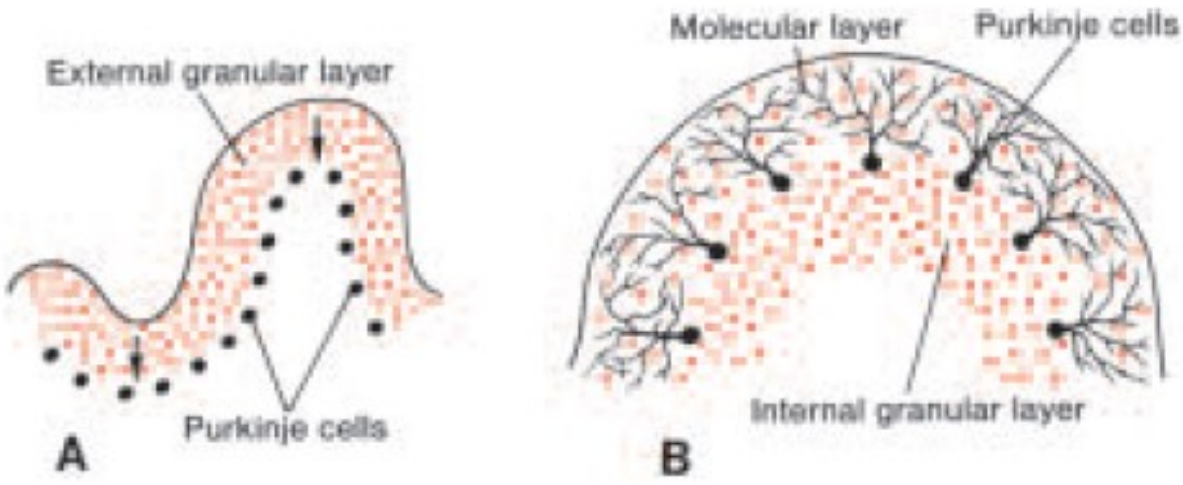


Figure 2.18: Phases in cerebellar cortical development. **A**. A proliferative layer from which GCs arise from the EGL on the exterior of the cerebellum. **B.** Postnatal cerebellar cortex depicting PCs (Sadler, 2014).

Usually after the last cell division, cerebellar GCs stay in the EGL for at most 48 hours before beginning radial migration through the ML, the whole essence of this waiting time is not known (Komuro and Rakic, 2001). Early studies have jointly established the common concept using 3-H-thymidine labeling that cerebellar GCs are derived from the EGL and travel radially from the ML to the IGL along radial processes of BG where they stay in the cerebellum of adult after their last cell division. Granule cells take below 5 hours to travel across the ML and are subsequently distributed in the IGL. Intrinsic mechanisms control GC migration in the cerebellum and there is a continuous movement of these cells even after entering the IGL for a programmed period except they are instructed by an external signal to cease (Fujita *et al*., 1966).

In living slice preparations, real-time observation shows that after the terminal mitosis, future GCs wait in the EGL between 24 - 48 hours (Komuro, 2001). Accordingly, in that period, many postmitotic cells are arranged in the horizontal plane, and both straight processes become transformed into parallel fibres, this is rightly deduced from the Golgi procedures (Cajal, 1911).

Taking a look at the configurational adjustments and activities of GCs that have undergone cell division located within the EGL using a confocal microscope reveals two transverse processes that are not the same which are assembled equivalent to the longer axis of the folium, the GCs that have divided begin to move tangentially along the longer process. The speed and the cell composition get shifted steadily with their arrangement in the EGL. Midway into the EGL, how the cells move tangentially is very rapid in particular in cells with two not too long parallel branches.

As GCs extend and lengthen their larger branches, movement is decreased. At the border of the EGL, cell body sends few processes that go downwards into the ML. As soon as the resting moment is passed, GCs quickly send one straight branch and begin the switch from tangential to radial movement, modifying their round cell body into a straightly elongated fusiform state (Komuro, 2001).

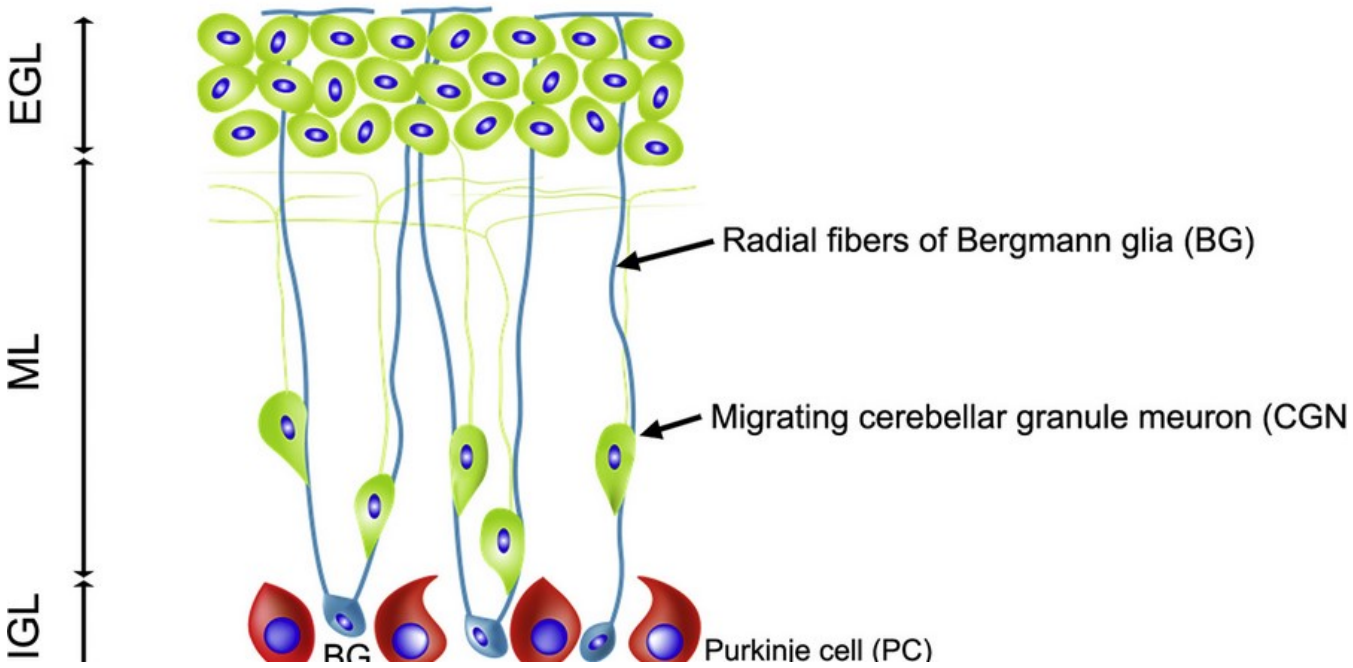


Figure 2.19: Gliophilic migration using radial fibers of Bergmann glia (Source: Sciencedirect.org)

2.14 CYTODIFFERENTIATION

Immediately the cells get transformed from proliferative cells in the EGL, DNA synthesis ceases. The cells leave this layer and build up instantly beneath it thus forming the postmitotic layer. This manner of cytodifferentiation has unusual resemblance to the ones available in neuroblast differentiation that is in the matrix system of the 4^{th} ventricle (Fujita, 1964).

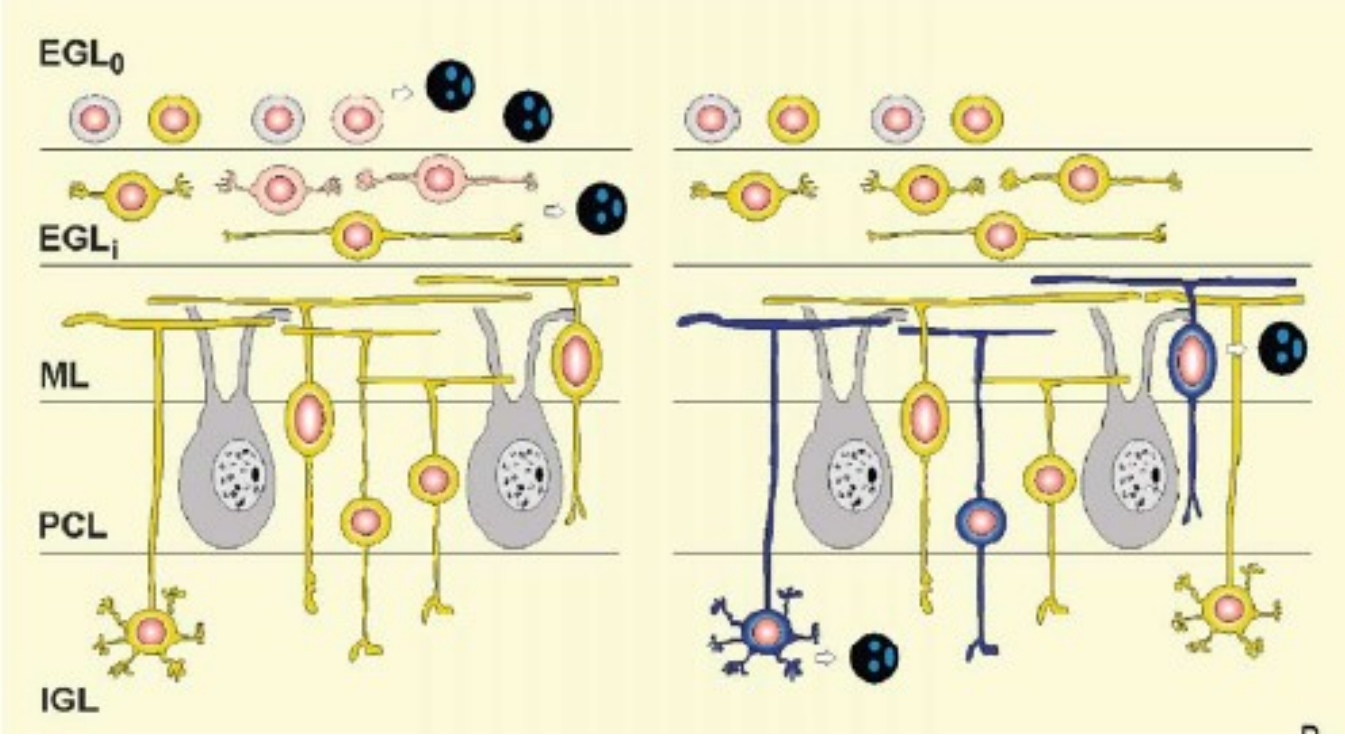
2.15 DESTRUCTION OF THE EGL

Vigorous increase in cellular quantities is observed in the EGL of most mammals shortly after delivery (Fujita *et al*., 1966). This zone is particularly susceptible to exposure to X-ray, certain substances and contaminations (Herdon *et al*., 1971). Destruction of this layer causes consequent anomalous cerebellar cytoarchitecture (Hopewells, 1974). It is imperative to say that when proliferation is disturbed, migration is also distorted. This has been confirmed with irradiation using x-rays; cisplatin, a cytostatic drug which obstructs mitosis in the maturing rat cerebellum (Scherini and Bernocchi, 1994), and methylmercury (Ponce *et al*., 1994).

2.16 CELL DEATH AND APOPTOSIS IN THE EXTERNAL GRANULAR LAYER

In neural development apoptosis is an imminent process. It is presumed that during neurogenesis almost 50% of the neurons generated die before the CNS completes its maturation, and almost all categories of neurons are affected by this process because almost all categories of cells are generated surplusly as the organism grows. Apoptosis has been identified to be an indispensable activity in development where it seems to be basic to controlling the closing amount of neuron types and glia produced in the CNS and PNS. It is becoming largely established that apoptosis is also accountable for the neuron deficit related with physiological aging (Kaufmann *et al*., 2001). These largely-produced neurons are significantly trimmed down in the process of naturally occurring neuronal death (NOND) when apoptotic machinery is activated.

In various parts of the CNS, it seems that two successive phases of loss of cells are observed: the former occurs primarily at the start of neurogenesis and is not too associated with synaptic formation, whereas the other is associated with the organization of nascent cells that have undergone cell division. In the two types, the death of the neurons is the apoptotic type (Bähr, 2000).



EG_0	External granular (mitotic layer)
EG_1	External granular (migratory layer)
ML	Molecular layer
PCL	Purkinje cell layer
IGL	Internal Granular layer

Figure 2.20: Pathways leading to death relating to multiplication, differentiation, and movement of granule cells (Lossi *et al*., 1998).

In the cerebellum, postnatal neurogenesis goes alongside an active stipulated death of cells to achieve a balance among the nerve cells, especially the GCs and the PCs. Different categories of death might have an effect on neurons, namely apoptosis (Kerr *et al.*, 1972), with fundamental principal characteristics being DNA disintegration, condensation of genetic materials, cell breakup or contraction, and turning on of special cellular proteases. Apoptosis takes place in the granule cells in two separate stages of differentiation, maturation, and migration: the first part takes place in the EGL and does not depend on the formation of synapses, the other occurs in the IGL. The first segment of GC apoptosis is quick and important for the overall control of the growing pre-mitotic group of these cells; the next segment takes place over an extended period and match up to the phase of shaping of the synapses in the CC (Lossi *et al*., 2002). Connections between the Purkinje neurons and GCs is key to the functioning of the cerebellum, and it is generally admitted that both groups of cells manipulate each other and these influences are basic to the control of cellular existence or loss. Imperative for proper cerebellar maturation is a correct balance being maintained between neurogenesis and apoptosis, to the extent that equilibrium is modified in a number of mutations that are unplanned for which affect the cerebellum of mice causing ataxias.

Cell death may be nuclear or cytoplasmic and this can recommend cellular processes that could cause cell death (Cunningham, 1982). Parts of the brain where a high percentage of dying cells are seen include the posterior lobules and vermis (Altman, 1969).

Orchestrated death of cells is a fundamental course seen in various types of cells, neurons inclusive. One major use of apoptosis in the emerging CNS is the removal of excess cells and formation of proper synapses (Oppenheim, 1991).

Early accounts of loss of cells in the growing cerebellum were pinned on nuclear pyknosis and disintegration. The proportion of cells that were dead compared to mitoses reached a maximum at 1% on the PND 10 (Deo *et al*., 1979). The capacity of the IGL reached its peak on PND 25 days in rats. The EGL equally fades away simultaneously, and later reduces by 20%, attaining size of adult by PND 50 (Heinsen, 1977). In normal rats, the pyknotic index in the IGL was <0.1% on PND10, 14, and 21 with dead cells seen more near the WM on PND 10 and near the PCL on PND 21 (Rabié *et al*., 1977).

2.17 TRYPTOPHAN

Tryptophan (L-Trp) is a unit of protein of the twenty-two standard ones (Atkins and Gesteland, 2002) already eastablished and numbered as part of the nine essential amino acids required in the human diet.Trp was first discovered and stated in 1901 by Frederick Hopkins by hydrolysing casein, a whey protein found in milk.In the benchmark genetic code for amino acids, it is programmed as the codon *UGG.* The L-stereoisomer of Trp is available and utilized in enzyme or structural units of substances responsible for body building. The unique configurational feature of Trp seems to be that it has an indole group responsible for its activities. It is reported to have growth effects on mice. It has a molecular formula of $C_{11}H_{12}N_2O_2$ and a molar mass of 204.23gmol. Ability to dissolve in water increases as temperature rises: 0.23g/L at 0^{o}c, 11.4g/L at 25^{o}c, 17.1 g/L at 50^{o}c and 27.93g/L at 75^{o}c.Elevated levels of Trp presence in the brain is indicated in increased brain serotonin (5HT) and 5- Hydroxyindoleacetic acid (5HIAA) synthesis (Feurte *et al.*, 2001).

In animals, Trp is required in the nutrition of animals because of its broad range of physiological activities. First of, it is a material required for production of protein (Ruan *et al.*, 2014), it also enhances the consumption of food in poultry and livestock (Woodger *et al*., 1979), it is essential to improve development (Cortamira *et al*., 1991) and one of the factors in the synthesis of chemicals that encourages growth or influences how cells and tissues functions (Le Floc'h *et al*., 2008), likewise, it is well known that lack of Trp reduces the secretion of antibody in rats (Gershoff *et al*., 1968) elaborating its role in immune system not neglecting that it is a composition of all body building substances, Trp is antecedent to the production of serotonin which is neurotransmitter and is engaged in the CNS to slow down violent behavior and adjust responses to stress in social and environmental adaptableness (Dong and Zou, 2017).

During embryogenesis, the role of 5-HT – a product of Trp breakdown has been reported in the activities of nerve growth and gradual transformation. The influence of Tryptophan on the embryological growth of the cerebellum has been investigated. This is important because neurogenesis commonly occurs in the cerebellum. When protein is restricted in the diet it reduces the rates of cell differentiation which leads to distruption in cell migration and synaptogenesis.

It is probable that deficiency in monoamines such as Trp leads to structural alterations in function as the brain develops. Critical periods of development and brain region dictate the

differentiation of neurons and glia and formation of synapses. Maturation of the cerebellum takes place after birth, and the cerebellum is more susceptible to various negative local factors than the other parts of the brain when development after birth begins (Shambaugh *et al*., 1996).

Absorbed tryptophan move around freely or attached to albumin as it circulates in the blood stream. Tryptophan is broken down by three discrete methods: (a) the production of kynurenine derivatives – main route, (b) the synthesis of serotonin (Schroecksnadel, 2006 and Chen, 2009) and (c) the biosynthesis of proteins.

Though the liver utilizes more than almost all of Trp found in the plasma, a definite quantity departs from the liver to go into the brain. While in the brain, enzyme tryptophan hydroxylase (TPH) changes Trp to 5-hydroxytryptophan (5-HTP), and another enzyme, 5-hydroxytryptophan decarboxylase converts it to serotonin.

Further to its indispensable function in protein synthesis, L-Trp is the predecessor of various metabolites that are physiologically formed in the process of its breaking down using four different pathways, three are of negligible importance, while the fourth, the kynurenine pathway (KP), does almost all the entire Trp breakdown (Bender, 1983; Badawy, 2002). The vital yields of the 3 less important pathways are: serotonin in the brain and melatonin in the pineal gland through hydroxylation; tryptamine through decarboxylation; and indolepyruvic acid (IPA) through transamination.

2.18 METABOLISM OF TRYPTOPHAN INTO ITS METABOLITES

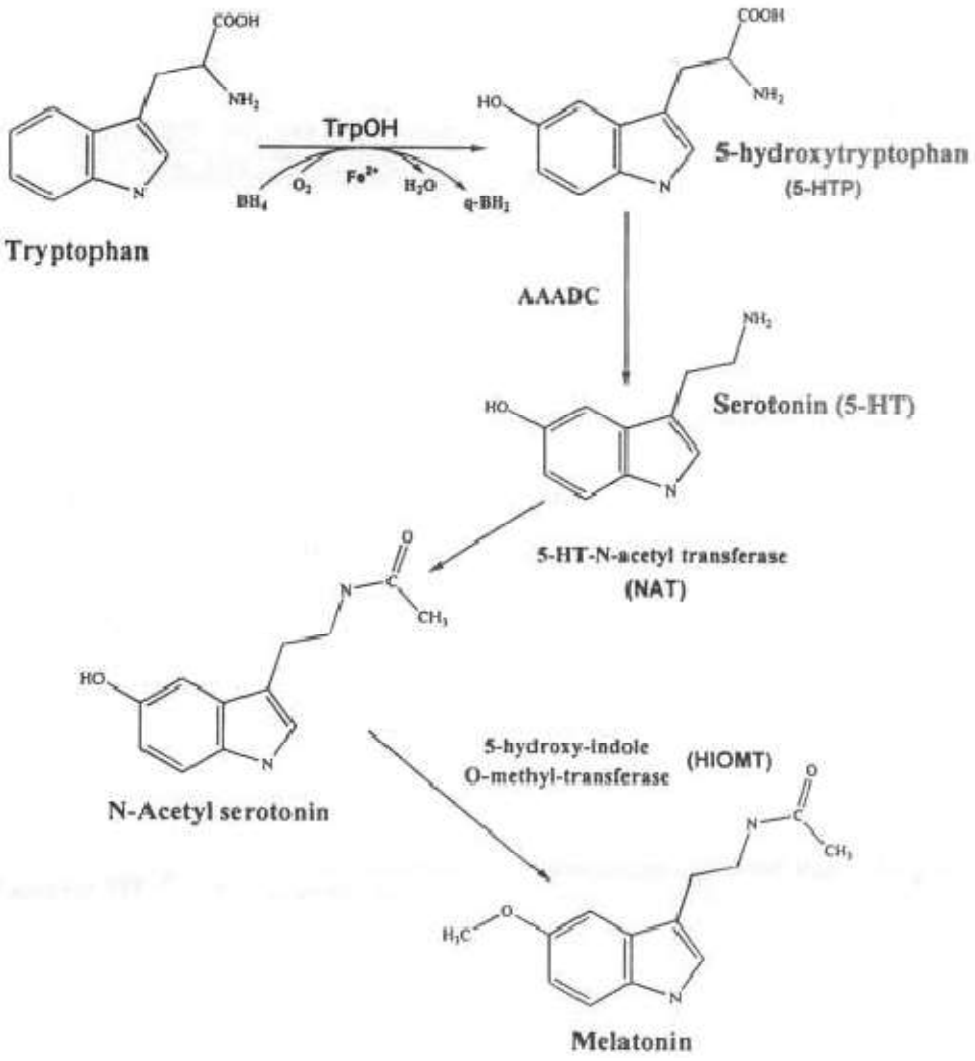


Figure 2.21 L-tryptophan metabolism into its products: melatonin and serotoninn. (Source: IUPAC-IUB Joint Commission on Biochemical Nomenclature, 2007).

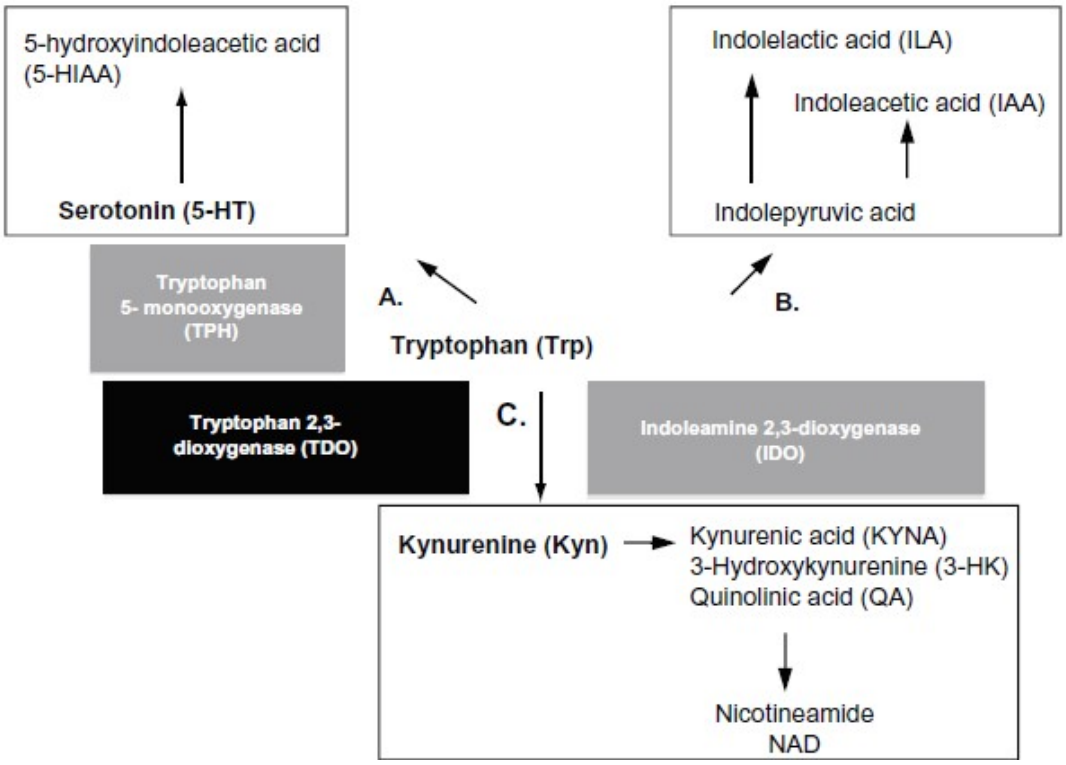


Figure 2.22: Ways by which Tryptophan is broken down showing the three primary methods. A: Serotonin pathway B. Transamination pathway. C. Kynurenine (Kyn) pathway (Funakoshi *et al.,* 2011).

2.19 Functions of Trp

Tryptophan is outstanding in its variety of biological functions (Moffat*et al.*, 1986). It is an essential part of body building foods and vital in personal diet for regulating a stable balance of nitrogen and the preservation of body weight (Morroni 1990; Fiorucci and Cavalheiro, 2002). Trp is antecedent to the neurotransmitter serotonin and carries out a crucial task in brain function and associated mechanisms that regulated these functions (Liang and Song, 2005). Similarly, Trp is frequently used as an essential starting substance in the manufacturing of a number of drugs (Altria *et al.*, 1996). It is commonly used in the management of schizophrenia, hypertension and gloominess (Budaveri *et al.*, 1996). Tryptophan is extensively employed in the manufacturing of edible substances where it is put in nutritional products to fortify foods so as to preserve the protein quantity in food and effect corrections where there are dietary deficiencies. The indole moiety of the organization of proteins can be studied using Trp (Cioni and Strambini, 2002). In protein biosynthesis, Trp acts as a building block. Trp is fundamental to the production of the following compounds:

- Serotonin
- Niacin
- Auxin

Kynurenines and serotonin which are metabolites of Trp are concerned with biological roles, such as protein and energy syntheses, higher-order brain functioning and immune regulation (Peters, 1991; Moroni, 1999). Trp is available in traces (<1%) in most sources of protein. For Trp to access the brain, there is a struggle with other large neutral amino acids (LNAA) using the same medium by which they are conveyed. Diets low in protein diets manipulate the proportion of Trp to LNAA in support of Trp, in order for additional Trp to enter into the brain. Including Trp in the food we eat can augment the proportion of Trp to other LNAAs, thus giving Trp an edge when competing to enter to the brain. The very essential enzyme - Tryptophan hydroxylase changes Trp to serotonin and is usually saturated halfway with Trp, such that an increase in the consumption of Trp adjusts the plasma Trp/LNAA proportion, controlling the levels of Trp in the brain and consequently regulating serotonin production and breakdown.

2.20 Enzymes involved in Trp Metabolism

The Trp metabolic pathways include the serotonin pathway, which comprises 5-HT and 5-HIAA whereby TPH is the initial enzyme in this pathway. Secondly, the transamination pathway is Trp metabolic method which consists of indolelactic acid (ILA) and indoleacetic acid (IAA). Simply, a minute amount of supplemental Trp is required in the production of units necessary for body building or in the seroronin and transamination pathways, and majority of Trp is broken down via the Kyn pathway mostly in the liver under standard states. The first enzyme in the pathway is tryptophan 2, 3- dioxygenase (TDO). Besides TDO, indoleamine 2, 3-dioxygenase (IDO) obtained from rabbit small intestine (Tone *et al.*, 1990) is assumed to play a perform outside the liver. These include reactions that are prone to immune indicators under extremely definite situations, such as autoimmune diseases and pregnancy.

Proofs exist that three factors affect concentration of brain tryptophan:

i. Alterations in the amount of a part of the free Trp in plasma (Knott and Curzon, 1972)

ii. Struggle between Trp and a group of neutral units of protein as they are being taken up by the brain, as studied in vitro (Kiely and Sourkes, 1972) and in vivo (Oldendorf, 1971).

iii. Efficacy of Trp as it is being taken up by the brain. This might have an effect on the action of central catecholaminergic systems (Hery *et al.*, 1974) and cyclic AMP (Tagliamonte *et al.*, 1971).

Pathway	Main products	Main function(s)
1 Decarboxylation	Tryptamine	Neuroactive amine
2 Transamination	Indolepyruvic acid	Anxiolytic, improves sleep, precursor of KA
3 Hydroxylation	5-HT (brain)	Behavioural control, cognition, emotion, mood, signalling pathways
	Melatonin (pineal)	Antioxidant, neuroprotection, circadian rhythm regulation
4 Oxidation	K	Precursor of Ks
	KA	Anxiolytic, neuroprotective, NMDA receptor antagonist; α-7 nicotinic acetylcholine receptor antagonist
	Anthranilic acid	Immune modulation
	3-HK	T-cell suppression
	Xanthurenic acid	Insulin binding
	3-HAA	T-cell suppression
	Nicotinic acid	Pellagra-preventing factor (vitamin B_3)
	QA	NAD^+ precursor, excitotoxic, NMDA receptor agonist
	Picolinic acid	Zinc binding
	$NAD^+(P)^+(H)$	Redox cofactors, cell viability and DNA repair

5-HT	Serotonin
K	Kynurenine
KA	Kynurenine Acid
3-HK	3- Hydroxykynurenine acid
3-HAA	3-Hydroxyanthranilic acid
QA	Quinolinic acid
NAD^+	Nicotinamide adenine dinucleotide

Table 2.2: Trp metabolic pathways and their main products (Badawy, 2015).

Table 2.3: Table showing amino acid wavelength and coloration

AMINO ACID	WAVELENGTH	COLOUR
ASPARAGINE	505nm	Light blue
ALANINE	602nm	Blue
ASPARTIC ACID	465nm	Purple
CYSTEINE	600nm	Blue
GLUTAMIC ACID	560nm	Purple
GLYCINE	525nm	Purple
HISTIDINE	460nm	Purple
ISOLEUCINE	580nm	Light purple
LEUCINE	590nm	Purple
LYSINE	450nm	Orange yellow
METHIONINE	525nm	Greenish yellow
ORNITHINE	570nm	Purple
PHENYLALANINE	545nm	Yellow
PROLINE	470nm	Yellow
PYRROLYSINE	455nm	Yellowish blue
SERINE	485nm	Blue
THREONINE	615nm	Bluish green
TYROSINE	530nm	Greenish blue
TRYPTOPHAN	565nm	Yellowish blue
VALINE	490nm	Greenish blue

$$\% \text{ Amino Acid (any one)} = \frac{\text{Absorbance of sample x Gradient Factor x Dilution Factor}}{10{,}000}$$

(Source: Method of Tryptophan Determination - Spectrophotometric analysis of amino acids, Schroeder *et al.,* 1990).

2.21 TRP AND EARLY BRAIN DEVELOPMENT

Trp is fundamental to hippocampal and cerebellar growth and function. Absence of it in the initial period development after delivery results in anomalous development in the cerebellum and hippocampus and produces reduced dendrite arborization, increase in cellular quantity and spine density in the dentate gyrus located in the hippocampus (Masaaki *et al*., 2010).

Besides its function in the hippocampus, Trp is assumed to be important in early cerebellar growth. This is inferred from a study that a diet deficient in Trp administered 5 weeks before pregnancy results in delayed BG growth and attending migration of granular cell in wistar rats (Del Angel-Meza *et al*., 2001).

2.22 TRP AND BLOOD BRAIN BARRIER

Trp singularly binds to albumin with about 10% available freely in plasma (Ruddick *et al*., 2006). Several factors influence its binding to albumin especially in the company of fatty acids, that are not esterified and exogenous causes, for instance drugs which are capable of dislodging tryptophan that are already bound. From all indications, Trp dissociates largely from albumin in the blood vessels found in the cerebrum, at a very high speed taking place when there is low cerebral blood flow which may be controlled in part by the sympathetic nervous system (Fernstrom and Fernstrom, 2006). From the circulating blood, free Trp is transferred from the blood brain barrier (BBB) through a transfer medium that is very competitive (L-amino acid transporter 1). This is co-used with a number of LNAA (Ruddick *et al.*, 2006). The carrier located in the blood vessels of the cerebrum is established to possess a more attraction for Trp than those located externally to the CNS.

Other LNAA vie for a common carrier in order that lesser quantities of those other protein units will enhance Trp transfer. Trp enters cells by processes that are not very clear although some evidence states the possibility of a transporter in serotonergic neurons (Herroro *et al*., 1983) and pinealocytes (Gutierrez *et al*., 2003) in the brain. It is doubtful at the moment if the transporters are important in brain cells like they are in the two-way substitute of Trp and Kyn in other cells (Kaper *et al.*, 2007).

Transport systems that make use of carrier proteins permit substances that dissolve slowly to go through the BBB. Molecules of glucose penetrate the brain from the blood using a transport protein. Glucose is the fundamental supply of energy in the brain. Glucose transport protein

(GLUT-1) is greatly augmented in endothelial cells of capillaries located in the brain. These carrier molecules allow the passage of glucose molecules across the BBB.

Various units of proteins such as isoleucine, leucine, tryptophan, valine, phenylalanine, histidine tyrosine and methionine which are also essentially needed in the body enter the brain as fast as glucose. This category of protein units is transferred into the brain by employing the leucine-preferring or the L-type transport proteins. There is competition between these compounds for gaining access into the brain. An increase in the quantity available in the plasma of one amino acid will slow down the absorption of others. Small neutral amino acids (SNAA) including proline, glycine and alanine are noticeably restrained in the brain from entering the brain. This group is the units that are not so required and is carried by alanine-preferring or A-type transport protein (Dash, 1997).

Trp entry into the brain across the BBB is typified by one-way-directional absorption which relies on concentrations of Trp and LNAAs that compete with it. The major transport mechanism for LNAAs is the carrier L-1 which cannot be produced in the CNS (Smith and Stoll 1998). As a result, the speed of invasion of unidirectional flow of these protein units absorption into the brain from the plasma is calculated using the Michaelis–Menten equation considering influx across capillaries after facilitated diffusion at L-1 (Smith *et al.,*. 1987a).

2.23 TRP AND PLACENTAL BARRIER

Trp transport through the plasmalemma is established to take place using various pathways in different cells. It has been stated that Trp given to gestational rats passes the placental barrier and stimulates an increment depending on varying doses of Trp concentrations in the brain, body and placenta *in utero* and this offers a similar rise in 5-HT and 5-HIAA concentrations in the growing brain (Arevalo *et al.*, 1991). A vital placental role is the uptake of tryptophan and its metabolism to serotonin and kynurenine metabolites, which are paramount for an increase in protein synthesis, immune function and fetal neuronal growth. The presence of mRNA and protein for the Trp and serotonin metabolic pathways has been reported in fetal growth restriction (FGR) placentae as described by (Murthi *et al*., 2016). L –Type transport system was reported as the primary method for the transport of L- Tryptophan across placental brush border that depends on the substrate specificity and trans-stimulation studies by Ganapathy *et al.*,(1986). L-Type

transport system is liable for L-tryptophan accessing the brush border membrane vesicles of human placenta (Christensen, 1979).

Using placental indoleamine 2, 3-dioxygenase, an enzyme which catabolises and controls feto-maternal immunology, the processes accountable for conveying L-tryptophan at the mother's- and fetal borders of the placenta is already established in membrane vesicles that are isolated. The uptake of L-tryptophan is significantly active osmotically at the brush border vesicle (Kudo and Boyd, 2001).

2.24 TRP AND BREAST MILK

In order to establish the level of Trp found in breast milk containing 6-sulfatooxymelatonin in babies that were breastfed all through for three months. Trp was made available through breast-feeding. In the development of brain serotonin-tryptophan level a neurobiological mechanism appear to be engaged (Cubero *et al.*, 2005 and Alegia *et al.,* 1996). Trp is available in large quantities in colostrum and breast milk but lacking in formula milk (Prescott, 1997).

2.25 ROLE OF SEROTONIN IN BRAIN DEVELOPMENT

In utero, serotonin functions in the mechanisms that regulate neuronal development. The quantity of serotonin available in the brain is reliant largely on the presence of Trp. In investigative study where corn (which is deficient in Trp) is used as a primary basis of body building food, a reduction in the production, absorption and breaking down of serotonin has been reported, especially in the rat brainstem and cerebellum (Beas-Za´rate *et al.*, 1988). When components responsible for body building in the diet is restricted, it decreases the rate at which cells differentiates and leads to hamperings in the migration of cells and formation of synapses. Additionally, a reduction of brain serotonin concentrations and serotoninergic activity is observed to take place when the brain is developing and when there is a deficiency of Trp in the diet.

2.26 IMPLICATION OF TRP IN CEREBELLAR DEVELOPMENT

Essential for cognitive development is an appropriate neural network development especially in the cerebellum and hippocampus. It has been reported that Trp also participates in the growth of these parts of the brain and their allied behavioral and mental activities. When Trp is lacking in

the diet especially during early postnatal development, it results in decreased spine density, dendrite arborization and multiplication of cells in the hippocampal dentate gyrus and nervousness and depression becomes evident (Zhang *et al.*, 2006). Kynurenine - a metabolite synthesized in primary neurons and glia is responsible for a rise in the synthesis of factors that make nerves to grow in astrocytes and has the capability to enhance the existence of young neurons in the mature hippocampus (Dong-Ryul *et al*., 1997). This is consequent to a report that a corn-based diet which is deficient in tryptophan started 5 weeks before exposure to male results in the slowing down of BG development and an attending granular cell migration.

Additionally, the liver TDO and new TDO alternatives that are produced and controlled in the brain may participate majorly in cerebellar and hippocampal development and function.

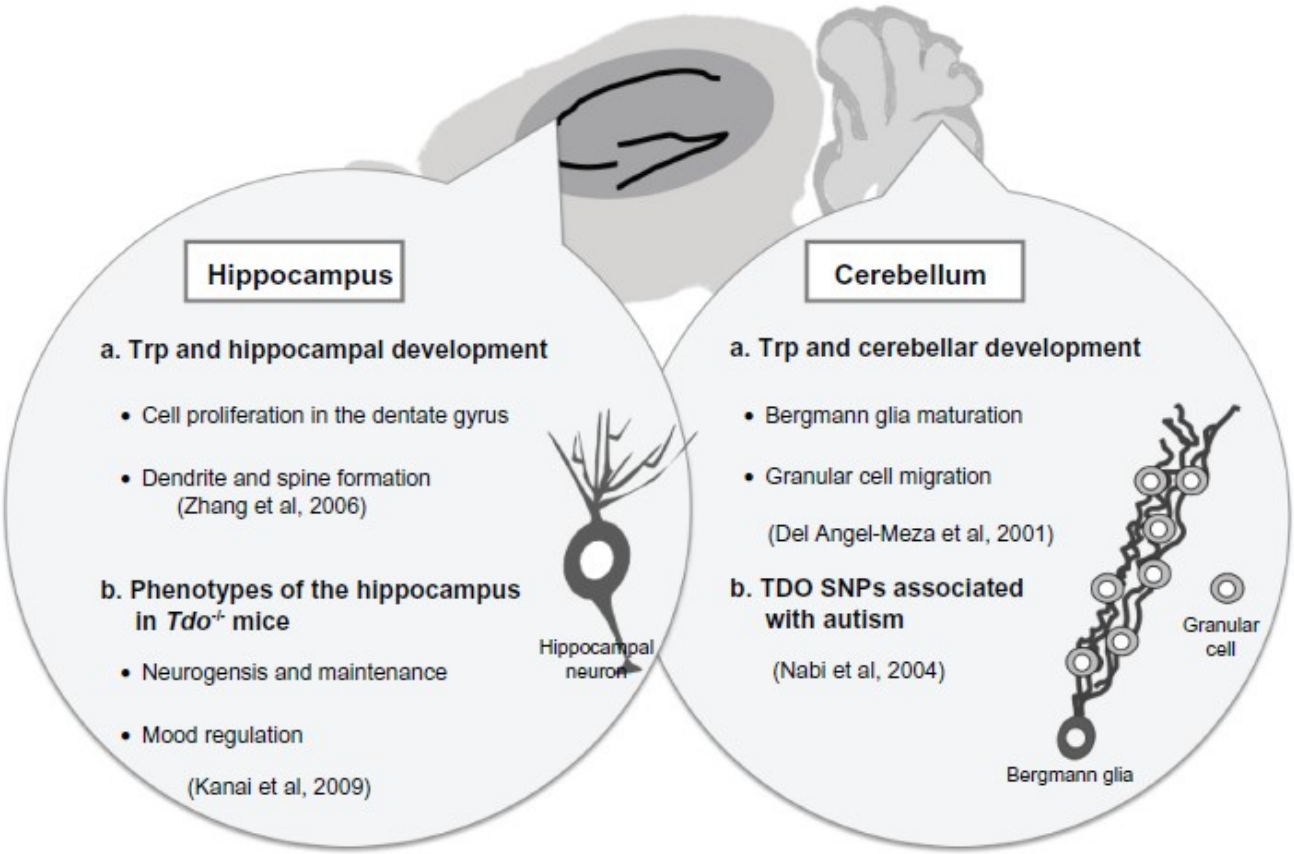


Figure 2.23: Role and significance of Trp in hippocampal and cerebellar growth (Source: Kanai *et al.,* 2009).

2.27 BLOOD SUPPLY OF THE EXTERNAL GRANULAR LAYER IN POSTNATAL CEREBELLUM

The number of GCs produced in the EGL is large. It therefore seems essential that this layer should have a rich blood supply to provide the necessary metabolites and that this vasculature should be capable of adapting to the eventual disappearance of the layer in early postnatal life. The cerebellum consists of a peripheral network of arteries which have radially oriented branches directed towards the ventricle, permitting a ventriculopetal blood flow.

The ventriculopetal arteries of the cerebellum are branches of the leptomeningeal vascular plexus which gives rise to long perforating arteries and short cortical arteries. Complementing the ventriculopetal system, but without connection to it, is a ventriculofugal blood flow, this is provided by arteries entering the cerebellum via the cerebellar peduncles and giving off branches to the deep nuclei (Van den Bergh & Van der Eecken, 1968). Arterioles penetrating the cerebellar cortex arise at right angles from the superficial arteries. They supply mainly the Purkinje cells and internal granular layer, ramifying to form a dense cortical capillary bed. In the white matter there is a less dense capillary bed which derives from the cortical bed and also from penetrating arterioles which extend below this. The molecular layer is poorly supplied with blood vessels, which form a thin, wide-meshed rete (Gillilan, 1969).

The meshes of the capillary bed of the cerebellar gray matter are radially oriented in the ML with smaller and irregularly polygonal branches in the internal granular layer. Arterioles penetrate to various depths and branch, thus supplying each layer with blood from all directions (Craigie, 1938). The pial membrane capillary network is very dense. In the very young animal, the most densely vascularised region was the pial membrane overlying the cerebellum. The concentration of capillaries in the pial membrane was so high that, in the photomicrographs of India ink and gelatin preparations, capillaries could not be individually resolved, and the capillary bed appeared as a dense mass. Nevertheless, the vasculature of the ML and EGL remained unchanged until the end of PND 21. Before this time it was strikingly poor in intrinsic capillaries.

The external granular layer, as a population of actively dividing cells, must depend on blood supply for the division and growth of its vascular bed in the IGL and WM.

The EGL remains almost free from intrinsic vasculature throughout its existence. On PND 21, the EGL finally eggresses with a change in the vascular pattern of the adult form. This shift is

not gradual, but takes place in a while. Since the external granular layer is so strikingly free of an intrinsic blood supply, its constituent cells, with their high proliferative activity, must require a source of metabolites, it is thus proposed that the vascular bed of the pial membranes provides this (Koppel *et al*., 1982).

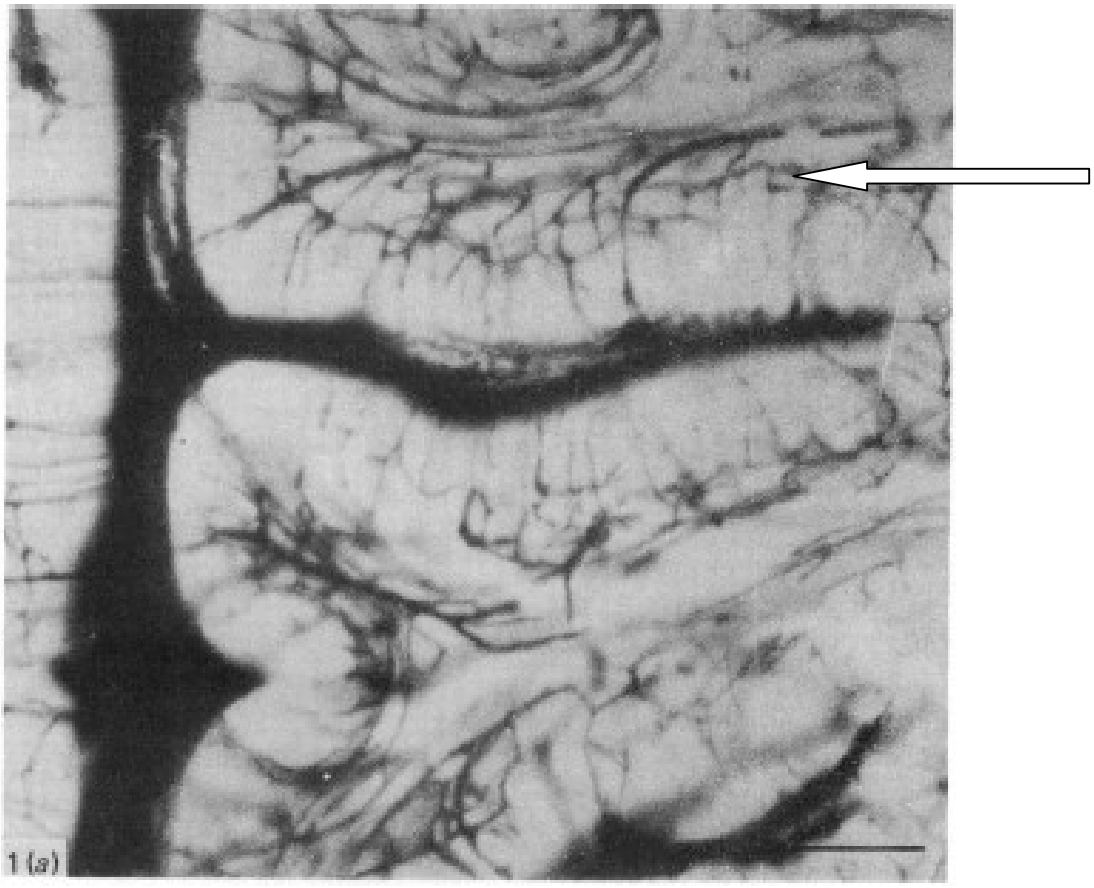


Figure 2.24a: Vasculature of a rat cerebellum aged 2 days indicating the fundamental intrinsic cerebellar capillary bed within the white matter. Arrows indicate capillaries.

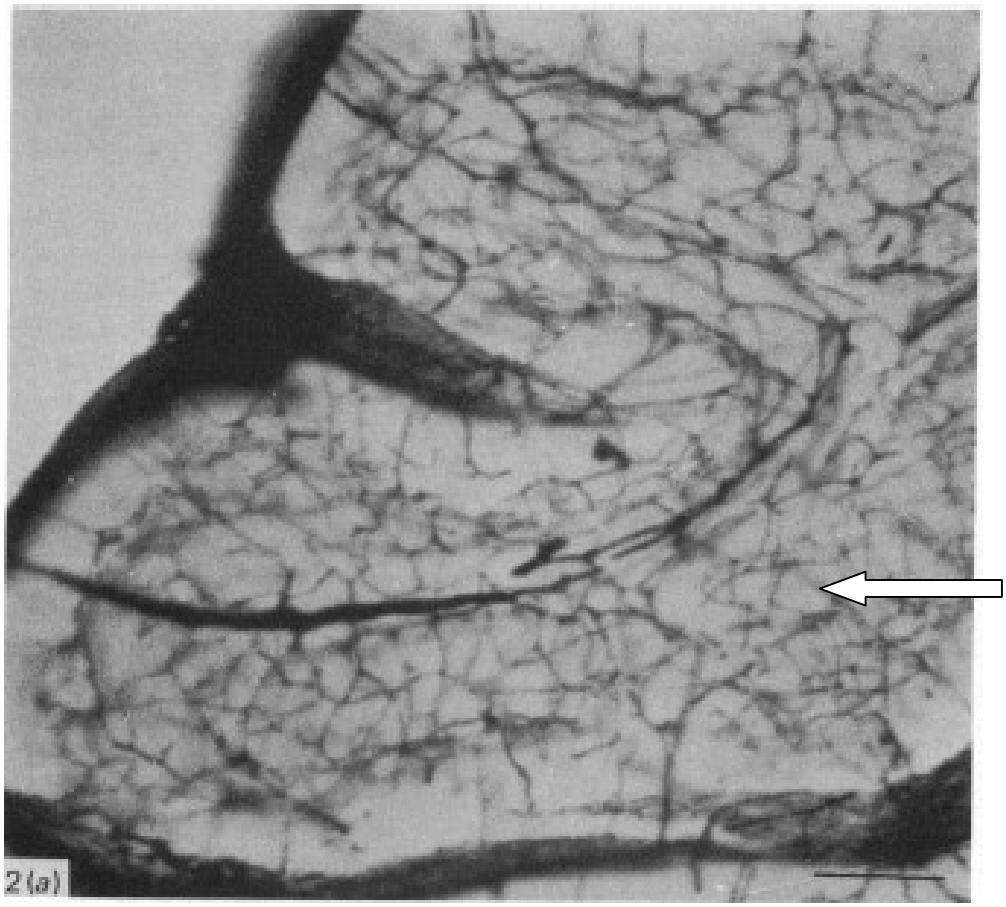


Figure 2.24b: Vasculature of the cerebellum of the 14 days old rat. The vasculature in the white matter/internal granular layer is denser. Arrows indicate capillaries (Source: Koppel, 1982)

2.28 RANGE OF DAYS FOR THE DISAPPEARANCE OF EGL

The total quantity of the IGL neurons develops post-natally in the EGL from PND 1 to PND 18 after transiting and developmental changes have occurred up to PND 21 after delivery. In Purdue-strain mice, the EGL vanishes on PND 18; the disappearance of EGL in the *dd* (dopamine deficient) strain takes place on postnatal day 20 (Fujita, 1967). Rakic and Sidman in 1973 reported postnatal day four to twenty as the time of EGL disappearance. The EGL is found on the CC until about 21 days of life (Altman 1971). Fujita (1967) and Altman (1972) stated that the rate at which cells of the EGL proliferate in the first two postnatal weeks is fast and that the proliferation reduces drastically in the following week, with an attending movement of the postmitotic cells and transformation of the EGL into the cerebellar cortex. This layer with active germinative properties dissolves gradually and fades away totally after 21 days after delivery.

2.29 MITOTIC INDEX AND CELL DIVISION

Growth of groups of cell takes place when cells exit interphase and mitosis and completes the cell cycle. As cells mature, they no longer have the capacity to go through mitosis and mitosis rarely takes place in some cells. For some others, it is easy to divide rapidly (Darbelley *et al*., 1989). It is anticipated that for those cells that hardly finish the cell cycle, they are in the latency of the cell cycle (G1). In a group of cells which rapidly divides, a reasonable sum of cells are in the state of active division of these individual cells. Mitotic indices show how to quantify cells in active division:

$$\frac{\text{number of cells in mitosis}}{\text{total number of cells}}$$

2.30 MITOTIC ACTIVITIES OCCURING IN THE EGL

Mitotic index is employed to estimate the rate at which cells proliferate within a group of cells. This is a proportion of the quantity of cells that are engaged in cell division and the entirety of the cells. Mitotic cells are located throughout the EGL from PND 0 through PND 8. The EGL of a mouse on PND 10 consists of four to eight lines of thickly collections of GCs. In the outermost two to three lines, cellular components that have divided are common and the mitotic index was 2.6%.

2.31 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) stains show how undifferentiated cells migrate inward and proliferate into differentiated cells during the first month of development, thereby forming the distinct layers of a cerebellum. In one week old mice, all progenitor neurons were located in the superficial layer of the cerebellum forming the EGL. By one month, no undifferentiated cells were left and distinct layers of the cerebellum had been formed.

2.32 KI-67 IMMUNOHISTOCHEMISTRY

Ki-67 immunohistochemistry is employed to observe the different changes in proliferative ability in the mammalian brain. It is an endogenous protein which is synthesized throughout all the segments of the cell cycle not includingG0. The cell multiplication antigen Ki-67 is principally synthesized in the cells of mammals that go through the process of cycling (Gerdes *et al.*, 1983), and is generally employed as an indicator for proliferating cells to know the status of tumours and can be used as an outstanding marker to decide the growth rate of a particular class of cells. The two variants of Ki-67 found in humans are with differing molecular weights of 320kDa and 359kDa (Gerdes *et al*., 1991), it is a protein that binds with nuclear DNA (MacCallum and Hall, 2000). One distinctive characteristics of this antibody is the possession of many sequences replicated (16 in human, 14 in mice) composed of a motif that is conserved role that is not known, the 'Ki-67 domain'. The levels of protein and locating Ki-67 locally are not constant in the cell cycle. Highest production is observed in G2 phase or when cell divides (Endl and Gerdes, 2000b). During interphase, structures that are fibre-like in regions that are deficient in fibrillarin enclosing nucleoli are formed around Ki-67 (Cheutin *et al.*, 2003). Ki-67 is generally needful for cells that multiply. Injection of antisense oligonucleotides that inhibit Ki-67 production restrained the multiplication of cells (Schluter *et al*., 1993). Normal proliferation rates in various cell lines results when Ki-67 expression levels are unperturbed (Zheng *et al*., 2006; 2009). Ki-67 is necessary to localize nucleolar granular counterparts to dividing genetic structures, thus performing a potential task in separation of the nucleolus amidst the new cells (Booth *et al*., 2014). Ki-67 has significant roles it plays in the biogenesis of ribosomes (Rahmanzadeh *et al.,* 2007), in line with the positioning of the nucleolus and remarkable roles in the proliferation of cells.

2.33 MOLECULAR FUNCTIONS OF Ki-67

Ki-67 interactors are been classified by gene ontology into two processes: ribosomal biogenesis and chromatin regulation. Interacting factors are further divided into classes that are implicated in the modification of chromatin, processing of pre-rRNA, transcription and protein translation, ribosomal subunit biogenesis, and linkages. These processes have proposed that Ki-67 may take part in biogenesis of ribosomes and in the regulation of chromatin (Rahmanzadeh *et al.,* 2007).

2.34 BCL 2 IMMUNOHISTOCHEMISTRY

B cell lymphoma 2 (Bcl-2) is an important factor in mammals which does not allow apoptosis to take place and its defence in opposition to apoptosis is connected to potassium ions [K^+]. Bcl-2 protein is proposed to contribute to neoplasia by repressing apoptosis but not being in itself oncogenic. Additionally, it has been reported that Bcl-2 protein enhances rebirth of the detached longer branch of nerves in the CNS and is also concerned in the control of gradual transformation of neurons (Zhang *et al*., 1996).

Generally, circulation of Bcl-2 and mRNA has been examined in the rat (Castre´n *et al.*, 1994). Bcl-2 is widely circulated in the young compared to the mature rodent brain, excluding parts where neurons can grow back after birth and areas where cells can be transformed into fully grown neurons, particularly in the olfactory bulb and dentate gyrus. On the contrary, PNS neurons incessantly produce high levels of Bcl-2 *in utero* up to maturity (Bernier and Parent, 1998).

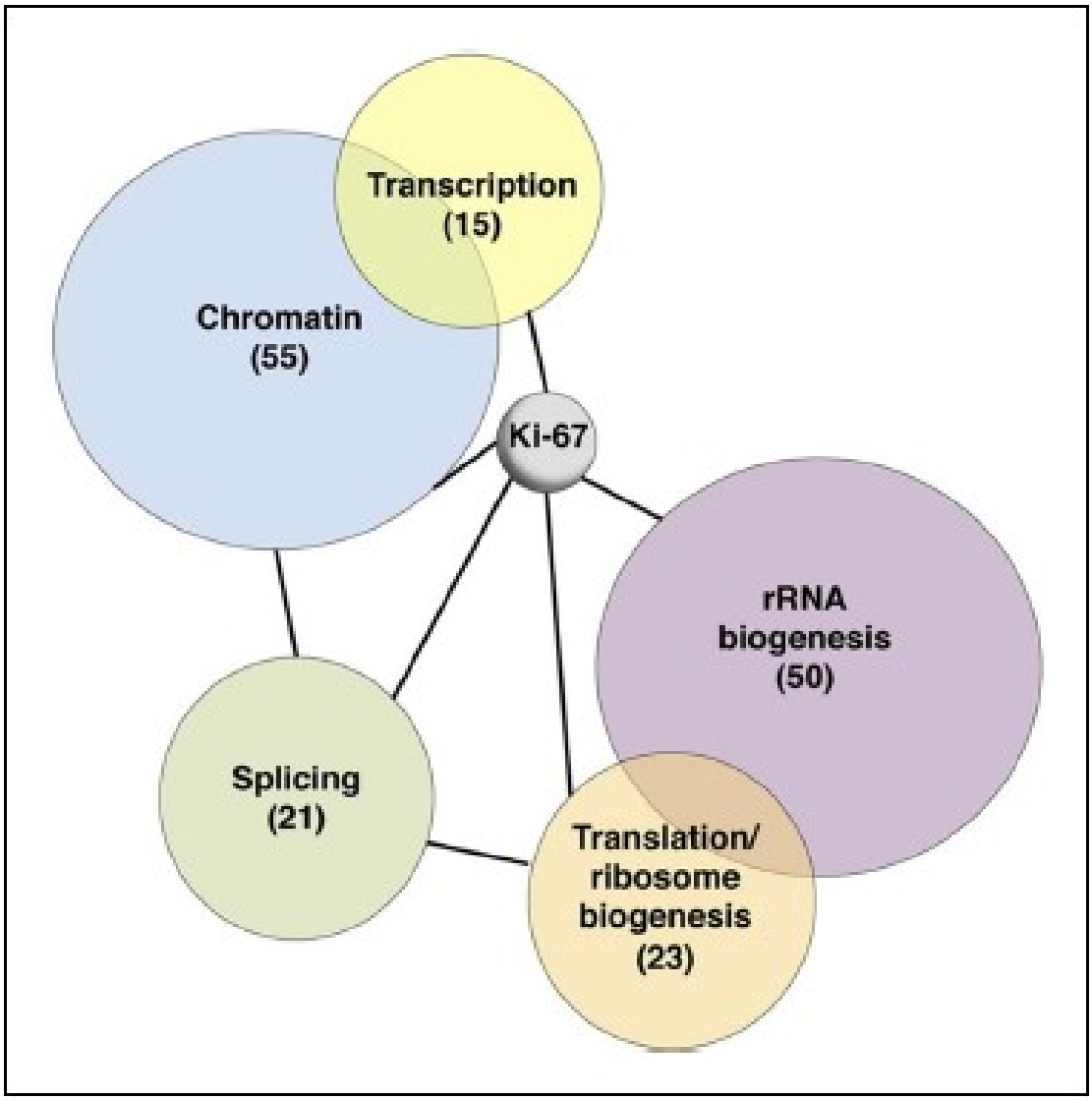


Figure 2.25: The relationship of Ki-67 with proteins implicated in chromatin control and processes in the nucleolus. Simplified string analysis shows network interactions between proteins associating with ki-67. (Sobecki *et al*., 2016).

2.35 Bcl-2 IMPLICATION FOR NEUROPROTECTION

It has been demonstrated that high potassium ion level leads to posttranslational building up of Bcl-2 in granule cells which allows for the defensive capacities of K^+ against apoptosis in young granule cells. In *in vivo* postnatal maturation of cerebellar cortex, apoptosis is the most frequent means of NOND and autophagy is now implicated in granule cells. While considering the level of maturation, cerebellar GCs die *in vivo* according to caspase mechanisms (Lossi *et al*., 2004). An integral protein which is incorporated into the lipid bilayer restrains apoptosis in some cell types and is produced by *bcl-2* proto-oncogene which includes cell lines from neurons after removing factors that can enhance growth (Batistatou *et al.*, 1993). When Bcl-2 is overexpressed *in utero* or after delivery, it drastically decreases the degree of naturally occurring neuronal death (NOND) in a vast number of neurons (olivary neurons and PCs inclusive) and many cells can be rescued from genetic lesions or damages (Zanjani *et al*., 1997). Neurons that were transfected that produced Bcl-2 in excess *in vitro* were shielded from stress that stemmed from apoptosis (Zhong *et al.*, 1993).

2.36 BCL-2 IN NEURONAL PROLIFERATION AND DIFFERENTIATION

The *bcl-2* proto-oncogene is secreted extensively as the CNS develops (Merry *et al*., 1994). Neurons die by apoptosis when they do not achieve their appropriate aim when they are formed at the onset as a result of inadequate factors that are aimed at the target (Davies, 1995). Bcl-2 rescues neurons by being a remedy to apoptosis and thus performs a major function in the determining of the shapes of neural tissues and in the formation of circuits in neurons (Williams and Smith, 1993). Neurons avoid apoptosis on their way to their destination by increasing Bcl-2 expression (Muller *et al*., 1997).

Synthesis of elevated quantities of Bcl-2 could indicate the presence of neurons that proliferate and neurons that have undergone mitosis and are not yet fully matured. It is also connected with the control of differentiation of neurons (Sato *et al.*, 1994). Neurons that express Bcl-2 exhibit a characteristic structural arrangement in the protein produced by neurons available in the germinative portions of the embryonic brain in rats. The salient facts concerning the function of Bcl-2 in the CNS include: (1) sites of intense Bcl-2 immunoreactivity occurs in regions where neurons maturate and not so much in proliferative centres in utero (Merry *et al.*, 1994); (2) in the PNS, this gene is produced all through adult life and neurons can start differentiation again

(Hockenbery *et al.*, 1991); (3) Bcl-2 is enriched in brain areas where neurons are produced continually, eg dentate gyrus, SVZ and olfactory bulb; (4) co-expression of Bcl-2 and nestin exist in SVZ neurons; (5) presence of a migrating guide for the neurons - glial septum; (6) the quantity of type A Bcl-2 immunoreactivity of neurons sharply reduces with brain maturation and age (Bernier and Parent, 1998); (7) There is a direct involvement of Bcl-2 in neural differentiation which assists nerve cells in fully grown CNS to regrow their long processes as a result of overexpression and (8) Nerve cells that express Bcl-2 stain intensely in the piriform cortex and amygdala have a morphology characteristic of immaturity seen in definite parts of the brain of primates in which is incomplete maturation.

CHAPTER THREE

MATERIALS AND METHODS

3.1 GROUPING OF ANIMALS

Forty adult locally bred female Swiss mice (28-45g) were used in this study. Mating was done by using one male mouse to five female mice. Postnatal day (PND) 0 was taken as the day of delivery. After delivery, each dam was housed together with her litter of pups in a separate cage and fed adequately as she nursed her pups. The animals were distributed into control group (Group I) was given distilled water, Group II was given 100mg/kg, Group III was given 200mg/kg, and Group IV was given 300mg/kg Trp solution orally (Santanna *et al.*, 1999). Dams in each group received single daily dose of Trp for varying days (1, 7, 14, 21) postnatally and the litters were sacrificed and processed for histology on different PNDs 0, 7, 14, 21 respectively.

A total of three hundred and twenty pups were used for this study. The mice and their litters were kept in plastic cages with wood shavings as beddings which were changed frequently. The animals were allowed access to sunlight and given water and diet as at when due.

The animal handling was done with care in compliance with the standard protocol. Authorization for the animals used was given by the Animal Care and Use Research Ethics Committee of the University of Ibadan (UI- ACUREC/19/0091).

Table 3.1 Animal (Pups) Distribution

	PND 0	PND 7	PND 14	PND 21
Group I	20	20	20	20
Group II	20	20	20	20
Group III	20	20	20	20
Group IV	20	20	20	20

3.2 DOSE OF TRP

Different doses of Tryptophan [Merck KGaA 64271 Darmstadt, Germany]: 100mg, 200mg, 300mg per kg body weight were given by stomach intubation to the lactating mothers for 21 days and these constituted group I (control), group II (100mg), group III (200mg) and group IV (300mg) correspondingly while the group I received distilled water. 200mg/kg was the standard dose given to the lactating mothers on a daily basis by stomach intubation according to Santana *et al*., 1999.

Dose: 100mg

1kg body weight ------100mg of Trp; average weight of pregnant mice is 32g

1000g -----100mg

$$32g = \frac{32g \times 100mg}{1000}$$

$$= 3.2mg$$

Dissolution of Trp

1000mg of Trp was dissolved in 100ml of distilled water

$$3.2mg \text{ of Trp} = \frac{3.2mg \times 100ml}{1000mg} = 0.32ml \text{ of dissolved Trp}$$

Dose: 200mg

1000g -----200mg

$$32g = \frac{32g \times 200mg}{1000g}$$

$$= 6.4mg$$

Dissolution of Trp

1000mg of Trp was dissolved in 100ml of distilled water

$$6.4mg \text{ of Trp} = \frac{6.4mg \times 100ml}{1000mg} = 0.64ml \text{ of dissolved Trp}$$

Dose: 300mg

1000g -----300mg

$$32g = \frac{32g \times 300mg}{1000g}$$

$$= 9.6mg$$

Dissolution of Trp

1000mg of Trp was dissolved in 100ml of distilled water

9.6mg of Trp = $\frac{9.6\text{mg} \times 100\text{ml}}{1000\text{mg}}$ = 0.96ml of dissolved Trp

The control group received water. The mice were monitored daily for any observable changes.

3.3 ANIMAL SACRIFICE AND DISSECTION

The anaesthestic agent used on the animals was ketamine injection which was given intraperitoneally (90mg/kg). Blood samples of the pups were taken before sacrifice for spectrophotometric test of Tryptophan. The animals were first decapitated before the subsequent removal of brain tissue for spectrophotometric estimation of tryptophan level. Transcardial perfusion was done for the animals with normal saline for three minutes. The brain tissues were removed and placed in 10% formalin as the fixative. Whole brain and cerebellum were blotted dry with filter paper and the weights taken using the Metler Analytical Balance.

3.4 TISSUE PROCESSING FOR HISTOLOGICAL STUDIES

The cerebellar tissues were prepared for histology through the following procedures: fixation, dehydration, clearing, infiltration, embedding, sectioning and staining. The tissues were trimmed to size. Fixation was carried out in 10% formalin in order to prevent tissue digestion (autolysis) by enzymes located in the cells or by bacteria and to maintain the molecular and structural composition and then transferred to70%, 80%, 90% alcohol and two changes of absolute alcohol for one hour.

Clearing of the tissues was done in xylene for one hour to replace the ethanol with a fluid that is compatible with wax.

Infiltration was carried out in four changes of molten paraffin wax to cause xylene to evaporate and to make the tissues transparent in an oven with a temperature of 60 degrees centigrade for 45 minutes each.

The tissues were embedded with an embedding mould to facilitate sectioning in molten paraffin wax. When the paraffin wax was cooled, it set as a hard block, which allowed for easy sectioning of the tissues.

The blocks were carefully placed on a wooden block and trimmed to the size needed with a knife. Using a Rotary Microtome, serial sections of 5 microns thickness were made. At a temperature of 30-40 degrees centigrade, cut slices were immersed in tepid water and placed on adhesive histological slides.

3.4.1 Haematoxylin and Eosin Staining

The staining was carried out using staining racks, each containing its own fluid. Sections were initially placed in xylene to dissolve away the paraffin wax. They were then:

i. transferred to two changes of descending grades (90%, 70%) of alcohol for five minutes each
ii. stained with haematoxylin for fifteen minutes
iii. differentiated in 1% acid alcohol (0.5% or 1% hydrochloric acid in 70% reagent grade alcohol) for 30 seconds (5 dips) for differentiating between nuclear and non nuclear structures. Acid alcohol is used to remove excess stain and define nuclei following hematoxylin staining in the H&E and PAP methods. It greatly improves reproducibility of staining results and helps achieve standardization.
iv. placed in running tap water for blueing for fifteen minutes
v. counterstained with eosin for one minute
vi. transferred to different ascending grades of alcohol. First to 70% alcohol for two minutes, then to 95% alcohol for two minutes and later to two changes of absolute alcohol at one minute each.

The slides were placed in two changes of xylene for one minute each. Mounting of sections was done using DPX as a mountant, after which the sections were ready for microscopic examination.

3.4.2 Cresyl Violet / Nissl Staining

Serial sections of the cerebellar cortex were laid on albuminized glass slides and left to dry over a slide warmer. The slides were taken through a process of rehydration before being immersed in 2% Cresyl violet acetate solution (Sigma-Aldrich, Germany) for 10 minutes, checking the coloration of the tissue, so it does not get too dark. They were dehydrated by passing through ascending concentrations of ethanol, 50%, 70%, 80%, 90%, 96% and 100% twice for 1 minute

each. If the tissue colouration was too dark, it was left longer in the alcohol, about 2-5 minutes instead of 1 minute.

The sections were then passed through 2 changes of xylene for 10 minutes each, coverslipped with DPX (BDH Chemicals Ltd, England) and allowed to dry.

3.4.3 Modified Golgi Staining

Fixative Solution

3% potassium dichromate---------- 60ml (3g in 100ml distilled water)

10% formalin----------20ml

Mix well and store at room temperature.

2% Silver Nitrate Solution

Silver nitrate----------2g

Distilled water----------100ml

Mix well and store at room temperature.

Procedure

Freshly harvested cerebella were deposited in 3% potassium dichromate for 5 days in a dark cupboard. Fresh chemical solutions were used for the tissues on a daily basis. The tissues were moved into 2% silver nitrate for 72 hours at 25^{o}C. Filter paper was utilised to wipe up the surplus solution before placing the tissues into silver nitrate solution. Solution of silver nitrate was changed. Tissues were infiltrated in molten wax for 30 minutes at 56^{o}C and then embedded in paraffin wax using molten wax dispenser Leica EG 1150H. Using Leica RM 2135 microtome, sections were cut at 60µm thick. Tissues were dehydrated through 70%, 80%, 90%, 100% alcohol and xylene for 5 minutes each and then mounted on albuminised slides (26x76x1.0mm), air dried for 10 minutes and coverslipped using DPX.

3.5 IMMUNOHISTOCHEMISTRY

Slides were put into plastic racks and labeled in pencil. Slides were then placed in the oven to warm the paraffin so that it melts and becomes easier to remove. Tissues sections were deparaffinized by placing slides into two turns of xylene for 5 minutes each and in equal parts of xylene and absolute ethanol. Cerebellar tissues were hydrated by passing slides through a

descending alcohol gradient, 5 minutes each in two changes of 100% alcohol, 90% alcohol, 70% alcohol, 50% alcohol and washed in distilled water. Slides were moved to 10mM citrate buffer, placed in the microwave and heated for 15 minutes. The tissue was microwaved open the binding site of the tissue so that the stain would be able to adhere to the tissue – Antigen retrieval. The container was removed from the microwave and the slides were allowed to sit in the warm buffer for 20 minutes or at 25^{o}c. Slides were then bathed in distilled water. Tissue slices were blocked in methanol and H_2O_2 for 20 minutes on a rocker. The methanol /hydrogen peroxide mixture blocks background staining but should not stay too long to prevent the stain from finding the binding site. Tissue slices were rinsed and rocked in 1% Phosphate Buffered Saline (PBS) three times each for five minutes. Using a PAP Pen, the tissue is circled and returned to the PBS solution. The PAP pen creates a hydrophobic barrier around the tissue to keep the antibody on the tissue, the slides are returned to PBS to disallow drying of the tissue. Tissues are blocked and rocked in 2% PBS milk for 60 minutes. Tissues were incubated in anti-rabbit Primary Ab (Ki-67[Anti-Ki67 antibody-ab15580, Abcam Company, USA] to show proliferating cells and BCL2 [Anti-Bcl-2 (E17) antibody-ab32124, Abcam Company, USA] to indicate apoptotic cells) with a dilution of 1 in 200 in a humidity chamber overnight in the cold room (+4°C) or at 25^{o}c (Ramos- Vara, 2011).

Primary antibodies were poured off and slides were washed in 1% PBS and secondary antibody (anti-rabbit), diluted in 1% PBS were added on the next day. Tissues were placed in secondary antibody for 60 minutes at 25^{o}c. Slides are put back in humidity chamber and incubated in (ABC) solution for 60 minutes and diaminobenzidine (DAB) was later put on the tissue and left to incubate for 5-10 minutes, DAB was poured off and washed in distilled water. Using alcohol, water molecules were removed from the tissues on the slides, to become lucid, slides were immersed in xylene and counterstained with hematoxylin or Trevigen Blue for 10-60 seconds before dehydration. Slides were coverslipped in the fume hood making sure that were no air bubbles (Ramos- Vara, 2011).

3.6 TRYPTOPHAN DETERMINATION

The level of Trp in the brain and blood samples was measured using Ninhydrin Chemical Reaction.

Ninhydrin Chemical Reaction for Spectrophotometric Determination of Amino acids

Principle: Combination of Ninhydrin with the amino acids form coloured complexes and amino acids present determine colour intensity.

Reagents: 0.1mol/l standard solutions of different amino acids (alanine, aspartic acid, leucine, isoleucine, lysine, methionine, glycine, threonine, valine, glutamic acid, cysteine, tryptophan, phenylalanine, ornithine, tyrosine, histidine, serine, proline, asparagines, pyrrolysine); acetate buffer at pH 5.5, methyl cellosolve (ethyleneglycolmonomethyl), 50% ethanol (v/v), hydrindantin, ninhydrin reagent (which is prepared by dissolving 0.8g of ninhydrin and 0.12g of hydrindantin in 30 ml of methyl cellosolve and 10ml of acetate buffer prepared fresh and stored in a brown bottle and 6MHCL (Schroeder et al., 1990).

Preparation of Sample by Hydrolysis

1g of well ground sample was weighed into a stoppered 350ml conical flask, 100ml of 6MHCL were added to the sample stoppered and heat in an oven or incubated for 16 hours to hydrolyze the sample. The mixture obtained was sieved over a double layered Whatman No. 42 filter paper into another 250ml conical flask and stoppered. The hydrolysate obtained was stored at - 4^{oc} if analysis is not done immediately.

Determination: 2 ml of the above hydrolysate was pipetted into a 30ml test tube. 10 ml of buffered ninhydrin reagent added, heat up for 15 minutes in a hot water bath, leave to attain a temperature of 25^{o}Cand 3ml of 50% ethanol was poured immediately.

0- 5µg/ml working standard amino acids was prepared from each standard solution of amino acids to get the gradient factor from the calibration curve for each amino acid. The working standards were heated with the buffered ninhydrin reagent as done with the sample hydrolysate above. The absorbances or transmittance of sample buffered heated hydrolysate and working standards were measured at the wavelength of colour developed by each amino acid.

3.7 MEASUREMENT OF GROSS AND MICROSCOPIC PARAMETERS

The fresh brain and cerebella were weighed using the Metler Analytical Balance. The stained sections were examined and the following parameters were taken: length of the layers (EGL, ML and IGL) of the CC and density of the external granular cells. Densitometry studies of the granule cells were done using the Motic Images Plus (Motic China Group, CO Ltd) Version 2.0 and Image J software application was used to determine the percentage positivity of the

proliferating cells. Images for densitometry were acquired with a Leica application suite 3.3.0 (Switzerland) with 40x objective.

3.8 HISTOLOGICAL STUDIES

Histological studies conducted on the cerebellum sectioned coronally at the paraflocullus were:

- Hematoxyin-Eosin cerebellar (cortical thickness and granule cell count)
- Cresyl violet (migration of radially oriented granule cells)
- Silver (Modified Golgi Method) (cytoplasmic processes)
- Ki67 – immunostaining (proliferating granule cells)
- BCL2 – immunostaining (Apoptotic cells)

Data obtained were analyzed using ANOVA at $\alpha_{0.05.}$

3.9 HISTOMORPHOMETRY

The following histomorphometric measurements were made using Motic Images Plus (Motic China Group, CO Ltd) Version 2.0 and Image J software application:

1. Overall quantity of cells in the EGL / unit area
2. Total of mitotic figures in the EGL / unit area
3. Total of dead /dying cells in the EGL / unit area
4. Mitotic Index = $\frac{\text{Total of cells that divided}}{\text{Overall quantity of cells}}$

3.10 STATISTICAL ANALYSIS

Quantitative data obtained from the morphometric measurements were subjected to statistical analysis. Sample means ± SD were obtained after running the statistical test to determine normal distribution and comparison among the various groups by Student t-test and one-way analysis of variance (ANOVA).

T-test is a type of inferential statistic used to determine if there is a significant difference between the means of two groups, which may be related in certain features. It is used as a hypothesis testing too; which allows testing of an assumption applicable to a population.

Analysis of Variance (ANOVA) is an analysis tool used in statistics that splits an observed aggregate variability found inside a data set into two parts: systemic factors and random factors. Analysts use the test to determine the influence that independent variables have on the dependent variable in a regression study. ANOVA is also called the Fisher analysis of variance and is the extension of the t- and z- tests (Fisher, 1992).

The ANOVA test allows a comparison of more than two groups at the same time to determine whether a relationship exists between them. The result of the ANOVA formula, the F statistic (F-ratio) allows for the analysis of multiple groups of data to determine the variability between samples and within samples

This was done after multiple comparisons using Tukey's post-hoc test.

Tukey Post –hoc Test also called Tukey's Honest Significant Difference Test is a post-hoc test based on the studentized range distribution. After running ANOVA with significant results, Tukeey's HSD is used to find out which specific groups' means when compared with each other are different. The test compares all possible pairs of means (Brillinger, 1984).

Statistical analysis was done using GraphPad Prism version 5.00 Windows Software (San Diego, California, USA). Confidence interval was calculated at 95% level.

CHAPTER FOUR

RESULTS

4.1 Animals

A total of 160 pups ranging from zero to three weeks old were used. Mothers were intubated orally with Trp and pups obtained Trp through breastmilk between 0- 21 days. Twenty five pups did not survive because they could not tolerate high doses of Trp (Group I - 0, Group II – 3, Group III – 7, Group IV - 15. Most of these deaths were due to excess analytical doses of Trp in their serum. A total of eighty mice served as controls**.**

4.2 Physical Observations

The pups were inspected for visible and observable developmental aberration. There was no physical gross abnormality. No behavioral abnormality was observed in the animals. The pups who received high dose of tryptophan looked weak and lost so much weight.

4.3. Brain weight (g)

The mean brain weight taken on PND 7 revealed a statistically significant gain in group II (0.25 ± 0.07)g in comparison with group I and group III (0.20 ± 0.03; 0.20 ± 0.05)g respectively while a significant loss of weight was reported in group IV (0.17 ± 0.03)g. On PND 21, significant weight loss was observed in group III and IV (0.28 ± 0.04*; 0.28 ± 0.02*).

Table 4.1 Brain Weight (g) of pups (Means ± SD)

	PND 0 N = 20	PND 7 N = 20	PND 14 N = 20	PND 21 N = 20
Group I	0.07 ± 0.01	0.20 ± 0.03	0.28 ± 0.02	0.33 ± 0.03
Group II	0.07 ± 0.01	0.25 ± 0.07*	0.29 ± 0.03	0.36 ± 0.04
Group III	0.07 ± 0.01	0.20 ± 0.05	0.28 ± 0.04	0.28 ± 0.04*
Group IV	0.07 ± 0.02	0.17 ± 0.03*	0.30 ± 0.08	0.28 ± 0.02*

*P < 0.05

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

4.4 Cerebellar Weight (g)

The average weight of the cerebellum of group I was measured and comparison was made with the treated groups (Table 4.2). On PND 7, group II (0.055 ± 0.01)g had a significantly increased cerebellar weight than group I (0.047 ± 0.01)g and the others with group IV (0.031 ± 0.07)g having significantly the least weight. Cerebellar weights for group II (0.064 ± 0.01)g and III (0.060 ± 0.01)g were higher than group I (0.053 ± 0.01)g on PND 14 while group IV still recorded the least. On PND 21, group VI also recorded the least weight among all the groups.

Table 4.2 Cerebellar Weight (g) (Means ± SD)

	PND 0 N = 20	PND 7 N = 20	PND 14 N = 20	PND 21 N = 20
Group I	0.019 ± 0.01	0.047 ± 0.01	0.053 ± 0.01	0.062 ± 0.01
Group II	0.019 ± 0.00	0.055 ± 0.01*	0.064 ± 0.01*	0.059 ± 0.01
Group III	0.022 ± 0.01	0.045 ± 0.01	0.060 ± 0.01*	0.062 ± 0.01
Group IV	0.016 ± 0.01	0.031 ± 0.07*	0.046 ± 0.01*	0.042 ± 0.01*

*P < 0.05

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.0

4.5 Total Serum Tryptophan Level (%)

There was no difference observed on PND 7 and 14. However, on PND 21, the mean serum Trp of Group II (0.020±0.012%) was significantly higher than group I (0.05±0.002%) and Group IV (0.002±0.001%) measured significantly lower than group I.

Table 4.3 Percentage Serum Trp (Means ± SD)

	PND 7 Mean n = 5	SD	P-value	PND 14 Mean n = 5	SD	P-value	PND 21 Mean n = 5	SD	P-value
Group I	0.003	0.001		0.004	0.001		0.005	0.002	
Group II	0.003	0.002	0.815	0.003	0.002	0.725	0.020	0.012	0.015*
Group III	0.003	0.002	0.672	0.003	0.001	0.247	0.011	0.005	0.053
Group IV	0.003	0.002	0.820	0.007	0.007	0.350	0.002	0.001	0.013*

*P < 0.05

Tryptophan level was measured as a percentage of the total serum amino acids in the pups.

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

4.6 Percentage Brain Tryptophan Level (%)

On PND 21, the percentage brain Trp of group II and group III were higher (0.0009 ± 0.0002%; 0.0012 ± 0.0002%) respectively than group I (0.0008 ± 0.0002%) and was significant. However, percentage level found in group IV (0.0007 ± 0.0002%) was significantly lower compared to group I.

Table 4.4 Percentage of Total Brain Tryptophan Level on PND 21 (Mean (%) ± SD)

	Group I n= 5	Group II n= 5	Group III n= 5	Group IV n= 5
Mean	0.0008	0.0009	0.0012	0.0007
SD	0.0002	0.0002	0.0002	0.0002
P-Value		0.0098**	0.0008**	0.0098**

*P < 0.05

** P < 0.01

Tryptophan level was measured as a percentage of the total amino acids in the brain of pups

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

4.7 Histological Studies

4.7.1 Cerebellar Cortical Width (mm)

The mean cerebellar cortical width (mm) was compared amidst the groups (Table 4.7). The mean cerebellar cortical width of group I on PND 7, 14 and 21 (0.19 ± 0.05; 0.28 ± 0.05; 0.26 ± 0.04 mm) did not show any significant difference from group II (0.18 ± 0.01; 0.27 ± 0.05mm; 0.29 ± 0.06mm), group III (0.20 ± 0.05; 0.27 ± 0.05; 0.27 ± 0.04mm) and group IV (0.22 ± 0.03; 0.25 ± 0.05; 0.22 ± 0.06mm) respectively.

Table 4.5 Cerebellar Cortical Width (mm) (Mean ± SD)

	PND 7 Mean n = 5	SD	P-value	PND 14 Mean n =5	SD	P-value	PND 21 Mean n = 5	SD	P-value
Group I	0.19	0.05		0.28	0.05		0.26	0.04	
Group II	0.18	0.01	0.704	0.27	0.05	0.746	0.29	0.06	0.522
Group III	0.20	0.05	0.656	0.27	0.05	0.807	0.27	0.04	0.946
Group IV	0.22	0.03	0.223	0.25	0.05	0.509	0.22	0.06	0.180

$P < 0.05$

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used

4.7.2 EGL Width (mm)

The mean EGL width was studied on PND 0, 7, 14 and 21 and comparisons were made between group I and the experimental groups. No significant difference was seen on PND 0 and 7 between group I (0.15 ± 0.02; 0.24 ± 0.03mm) and group II (0.15 ± 0.02; 0.24 ±0.02mm), group III (0.15 ± 0.03; 0.24 ± 0.04mm) and group IV (0.15 ± 0.02; 0.24 ± 0.04mm) respectively. However, on PND 14, group I (0.11 ± 0.00mm) and group IV (0.11 ± 0.00mm) measured lower when compared to group II (0.13 ± 0.01mm); group III (0.13 ± 0.01mm) and was found to be significant. No measurements could be taken on PND 21 for all the groups due to the disappearance of the cells.

Table 4.6: EGL Width (mm) (Mean ± SD)

	PND 0 n = 5	PND 7 n = 5	PND 14 n = 5	PND 21 n = 5
Group I	0.15 ± 0.02	0.24 ± 0.03	0.11 ± 0.00	----------
Group II	0.15 ± 0.02	0.24 ± 0.02	0.13 ± 0.01*	----------
Group III	0.15 ± 0.03	0.24 ± 0.04	0.13 ± 0.01*	----------
Group IV	0.15 ± 0.02	0.24 ± 0.04	0.11 ± 0.00*	----------

*P < 0.05

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

4.7.3 EGL Density (Cellular Density per 0.02mm^2)

The mean granular neuron count per mm^2 for PND 0, 7, 14 and 21 pups were compared between the groups. The mean EGL density for group I on PND 0, 7 and 14 (31.00 ± 1.23; 47.60 ± 2.51; 23.60 ± 8.02mm^2) were not different from group II (28.20 ± 4.32; 43.60 ± 3.36; 30.67 ± 2.52mm^2), group III (32.80 ± 4.32; 52.40 ± 5.55; 31.40 ± 2.51mm^2) and group IV (33.00 ±1.93mm 51.20 ± 6.54; 31.0 ± 2.15 mm^2) respectively. There was no granule cell count on PND 21.

Table 4.7: EGL Density(Means ± SD)

	PND 0 n = 5	PND 7 n = 5	PND 14 n = 5	PND 21 n = 5
Group I	31.00 ± 1.23	47.60 ± 2.51	23.60 ± 8.02	----------
Group II	28.20 ± 4.32	43.60 ± 3.36	30.67 ± 2.52	----------
Group III	32.80 ± 4.32	52.40 ± 5.55	31.40 ± 2.51	----------
Group IV	33.00 ± 1.93	51.20 ± 6.54	31.00 ± 2.15	----------

*P < 0.05

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used

4.7.4 Widths of the EGL, ML and IGL (mm)

On PND 0, no differences occurred between the EGL, ML and IGL measurements taken for group I as against group II, III and IV. The IGL width of group II (0.25 ± 0.07mm) on PND 7 was significantly lower than group I, group III and IV (0.26 ± 0.04; 0.35 ± 0.04; 0.34 ± 0.07mm). On PND 14, the width of the EGL in group I (0.11 ± 0.00mm) was significantly lower than group II (0.13 ±0.01mm), group III (0.13 ±0.01mm) and group IV (0.13 ± 0.02mm) respectively. ML width of group I (0.48 ± 0.12mm) was significantly greater than group III (0.32 ±0.04mm) and group IV (0.33 ± 0.04mm) on PND 14. On PND 21, IGL length of group I (0.48 ± 0.03mm) was significantly lower than group II (0.65 ± 0.08mm) (Table 4.8).

Table 4.8: EGL, ML and IGL Widths (mm) (Mean ± SD)

	EGL n= 5	P-value	ML n= 5	P-value	IGL n= 5	P-value
PND 0						
Group I	0.15 ± 0.02		0.12 ± 0.01		0.21 ± 0.02	
Group II	0.15 ± 0.02	0.788	0.12 ± 0.01	1.000	0.21 ± 0.01	0.854
Group III	0.15 ± 0.02	0.801	0.12 ± 0.01	1.000	0.22 ± 0.01	0.364
Group IV	0.15 ± 0.02	0.559	0.13 ± 0.01	0.433	0.20 ± 0.02	0.518
PND 7						
Group I	0.24 ± 0.03		0.15 ± 0.03	0.901	0.26 ± 0.04	0.916
Group II	0.24 ± 0.02	0.889	0.15 ± 0.02	0.639	0.25 ± 0.07	0.016*
Group III	0.24 ± 0.04	0.772	0.16 ± 0.02	0.831	0.35 ± 0.04	0.091
Group IV	0.24 ± 0.04	0.931	0.14 ± 0.03		0.34 ± 0.07	
PND 14						
Group I	0.11 ± 0.004		0.48 ± 0.12		0.50 ± 0.05	
Group II	0.13 ± 0.006	0.005**	0.32 ± 0.05	0.065	0.51 ± 0.09	0.779
Group III	0.13 ± 0.013	0.012*	0.32 ± 0.04	0.021*	0.57 ± 0.12	0.404
Group IV	0.11 ± 0.018	0.042*	0.33 ± 0.04	0.028*	0.53 ± 0.11	0.665
PND 21						
Group I	0.0		0.46 ± 0.03		0.48 ± 0.03	
Group II	0.0		0.53 ± 0.17	0.338	0.65 ± 0.08	0.021*
Group III	0.0		0.48 ± 0.06	0.331	0.50 ± 0.15	0.793
Group IV	0.0		0.46 ± 0.13	0.948	0.53 ± 0.09	0.394

*P < 0.05

** P < 0.01

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
* P<0.05	P value is significant at < 0.05
** P < 0.01	P value is significant at < 0.001

Table 4.9 Correlation Coefficients between the groups

PND	Layer	EGL	ML	IGL
0	EGL	--	--	--
	ML	--	--	-0.816
	IGL	--	-0.816	--
7	EGL	--	--	--
	ML	--	--	0.078
	IGL	--	0.078	--
14	EGL	--	-0.625	0.466
	ML	-0.625	--	-0.600
	IGL	0.466	-0.600	--
21	EGL	--	--	--
	ML	--	--	0.914
	IGL	--	0.914	--

Table 4.10 ANOVA: Tukey's Comparison of all Columns showing statistical differences between the groups

PND	P value	Parameters	Significance
0	<0.0001	EGL vs ML	Yes
		EGL vs IGL	Yes
		ML vs IGL	Yes
7	<0.0002	EGL vs ML	Yes
		EGL vs IGL	No
		ML vs IGL	Yes
14	<0.0001	EGL vs ML	Yes
		EGL vs IGL	Yes
		ML vs IGL	Yes
21	<0.0001	EGL vs ML	Yes
		EGL vs IGL	Yes
		ML vs IGL	No

**P<0.001

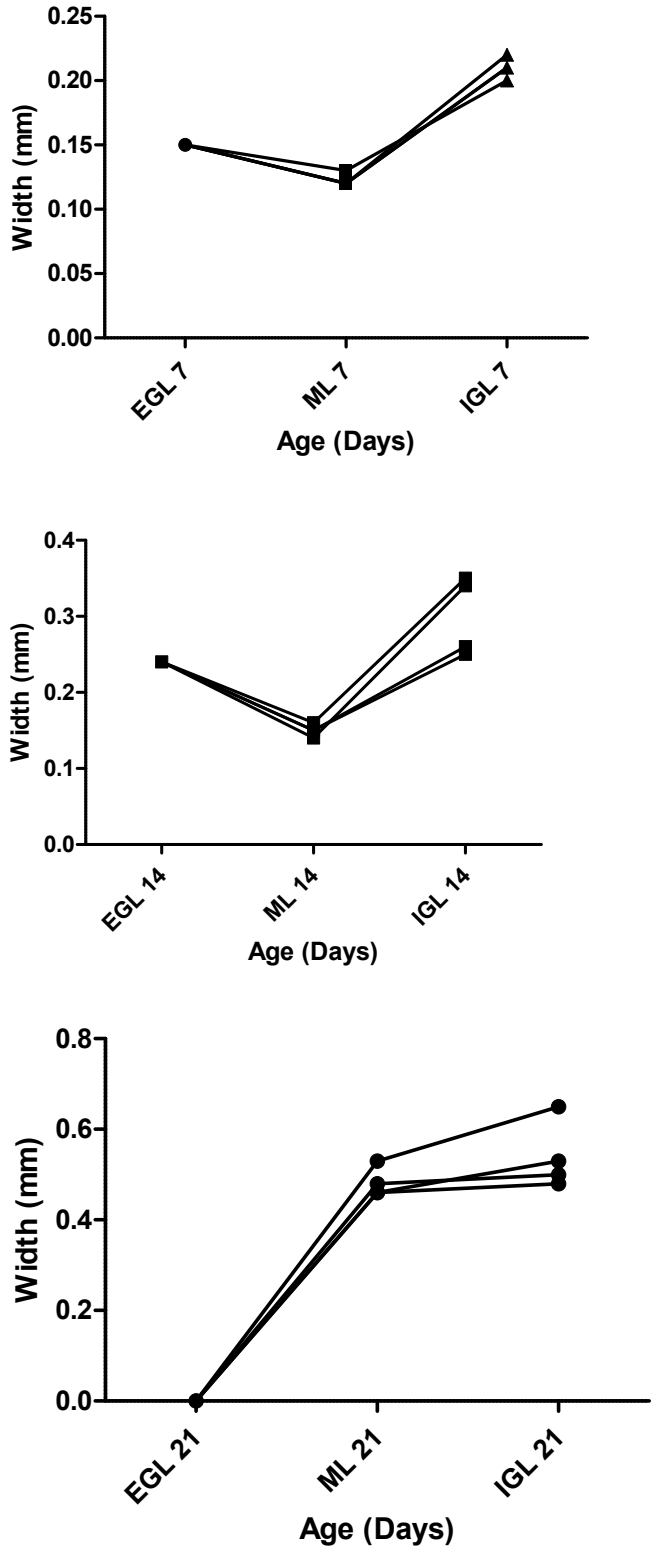


Figure 4.1 Charts showing the different widths on PND 7, 14 and 21

Table 4.11 Regression Analysis between the groups - EGL

Regression Statistics	
Multiple R	1
R Square	1
Adjusted R Square	0.666666667
Standard Error	0
Observations	4

Y= 0.15 + 0

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0	0	#NUM!	#NUM!
Residual	3	0	0		
Total	4	0			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.15	0	65535	#NUM!	0.15	0.15	0.15	0.15
EGL 21	0	0	65535	#NUM!	0	0	0	0

Table 4.12 Regression Analysis between the groups - ML

SUMMARY OUTPUT

Y=0.1556+(-0.0687)

Regression Statistics	
Multiple R	0.453989945
R Square	0.20610687
Adjusted R Square	-0.190839695
Standard Error	0.00545628
Observations	4

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.5458E-05	1.5458E-05	0.519230769	0.546010055
Residual	2	5.9542E-05	2.9771E-05		
Total	3	7.5E-05			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.155648855	0.046084052	3.377499313	0.077595654	-0.042634818	0.35393253	-0.04263482	0.353932528
ML 21	-0.06870229	0.095343481	-0.720576692	0.546010055	-0.478932178	0.3415276	-0.47893218	0.341527598

Table 4.13 Regression Analysis between the groups - IGL

SUMMARY OUTPUT

Y=0.2193+(-0.0172)

Regression Statistics	
Multiple R	0.16081688
R Square	0.025862069
Adjusted R Square	-0.461206897
Standard Error	0.009869843
Observations	4

ANOVA

	Df	*SS*	*MS*	*F*	*Significance F*
Regression	1	5.17241E-06	5.17241E-06	0.053097345	0.83918312
Residual	2	0.000194828	9.74138E-05		
Total	3	0.0002			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.219310345	0.04070472	5.387835688	0.032764921	0.044172069	0.394448621	0.044172069	0.394448621
IGL 21	-0.017241379	0.074823084	-0.230428612	0.83918312	-0.339179125	0.304696366	-0.33917912	0.304696366

4.7.5 Total Number of Cells in the Molecular Layer (per 4000 sqμ)

The entirety of cells in the ML was counted within and compared between the groups (Figure 4.1). The overall quantity of cells for the control group on PND 7, 14 and 21 (23.95 ± 5.21, 22.45 ± 5.37, 15.72 ± 1.41) was not significantly different from group I (23.32 ± 2.93, 18.8 ± 4.46, 23.67 ± 1.21); group II (24.24 ± 1.39, 18.34 ± 2.79, 16.6 ±2.84) and group III (23.28 ± 1.98, 19.96 ± 1.37, 16.82 ± 1.80) respectively.

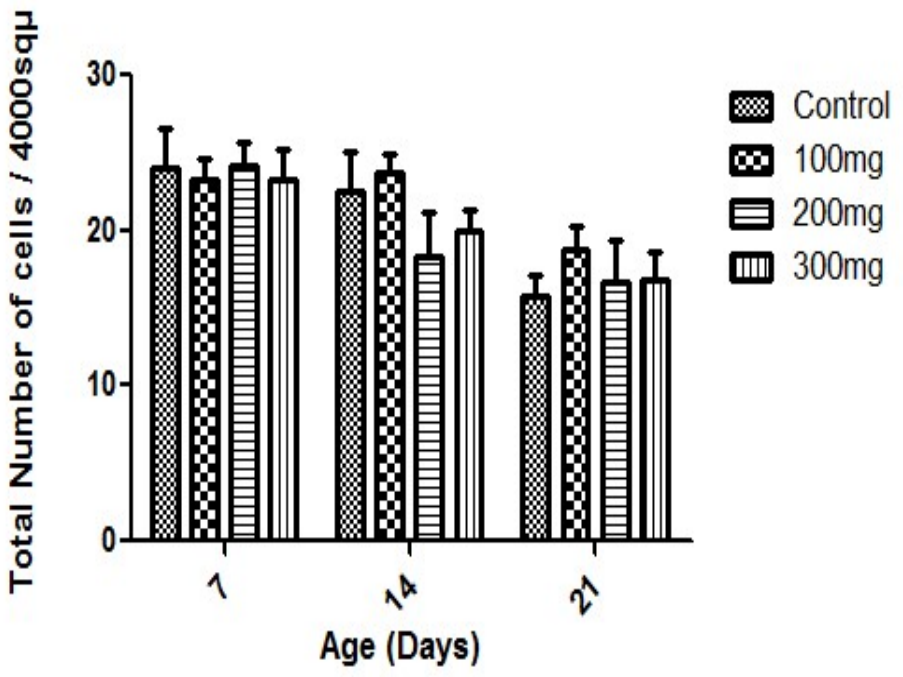


Figure 4.2 Chart showing Total number of cells in the ML on PND 7, 14, 21.

4.7.6 Total Number of Mitotic cells in the Molecular Layer (4000sqμ)

Mitotic cells were identified using Hematoxylin and Eosin stain. The nuclei of the dividing cells were in different phases of mitosis especially Telophase (Figure 4.2). The mean mitotic cell count per 4000sqμ for PND 7, 14 and 21 was done and compared between the groups. The mean mitotic cell count for group I on PND 7 (1.72 ± 0.22) was significantly lower than group IV (2.60 ± 1.73), while there was no difference in comparison with group II (1.85 ± 0.58) and group III (1.50 ± 0.47). On PND 14 and 21, no difference was observed in the mitotic cells between group I (1.31 ± 0.32, 0.6 ± 0.55) and group II (1.72 ± 0.49, 1.00 ± 1.00); group III (1.66 ± 0.42, 1.1 ± 1.14) and group IV (2.00 ± 0.79, 1.5 ±1.00) respectively.

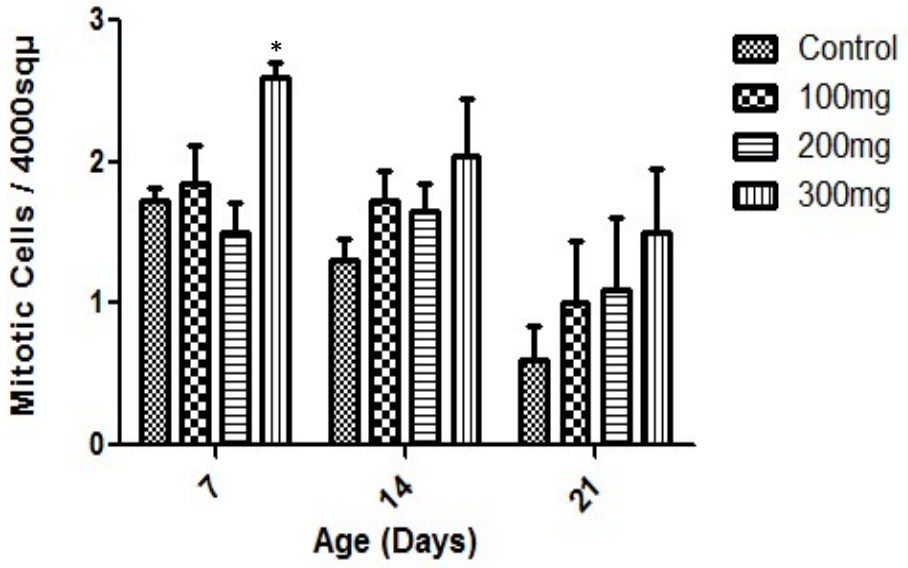


Figure 4.3: Chart showing means ± SD of mitotic cells in the ML on PND 7, 14, 21 (*P<0.05)

4.7.7 Total Number of Pyknotic cells in the Molecular Layer (4000sqμ)

Dying cells were identified using Hematoxylin and Eosin stained slides. Cells with clumped heterochromatin were counted as pyknotic cells. The count was done per 4000sqμ for PND 7, 14 and 21 and compared between the groups (Figure 4.3). On PND 7 and 14, no difference was observed when group I (1.32 ± 0.17; 1.38 ± 0.27) was compared with group II (1.47 ±0.41; 1.2 ± 0.11), group III (1.43 ± 0.26; 1.15 ± 0.22) and group IV (1.17 ± 0.26; 1.85 ± 0.51) respectively. However, group I (1.35 ± 0.29) was significantly the lowest when compared with group II (2.56 ± 0.83) and group IV (2.73 ±0.025) on PND 21.

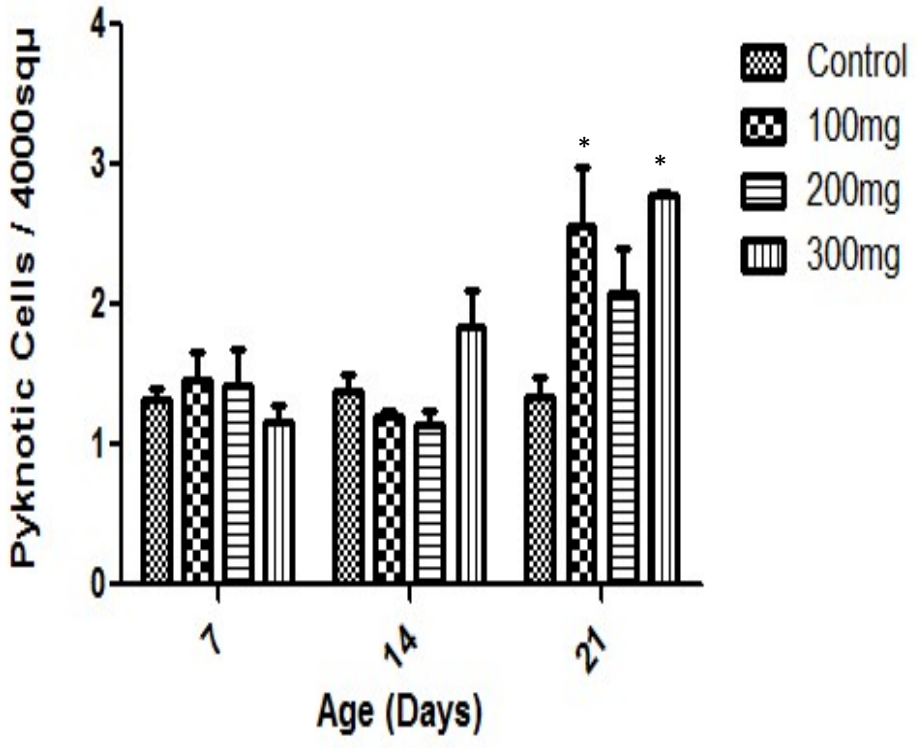


Figure 4.4: Chart showing means ± SD of pyknotic cell count in the molecular layer on PND 7, 14, 21 *P<0.05

4.7.8 Mitotic Indices Values (%)

Mitotic indices of the cells were obtained per 4000sqμ. Maximum mitotic activities were observed on PND 7, 14 and 21 across all the groups with group I recording the least values (7.2%, 5.8% and 3.8%) and group IV recording the highest values (10.9%, 10.3% and 8.9%) respectively.

Table 4.14: Mitotic indices (%) on PND 7, 14 and 21

	Group I n = 10	Group II n = 10	Group III n = 10	Group IV n = 10
PND 7	7.2	7.8	6.2	10.9
PND 14	5.8	7.3	9.1	10.3
PND 21	3.8	5.3	6.6	8.9

PND	Postnatal Day
N	Total number of animals used
%	Percentage of cells in mitosis

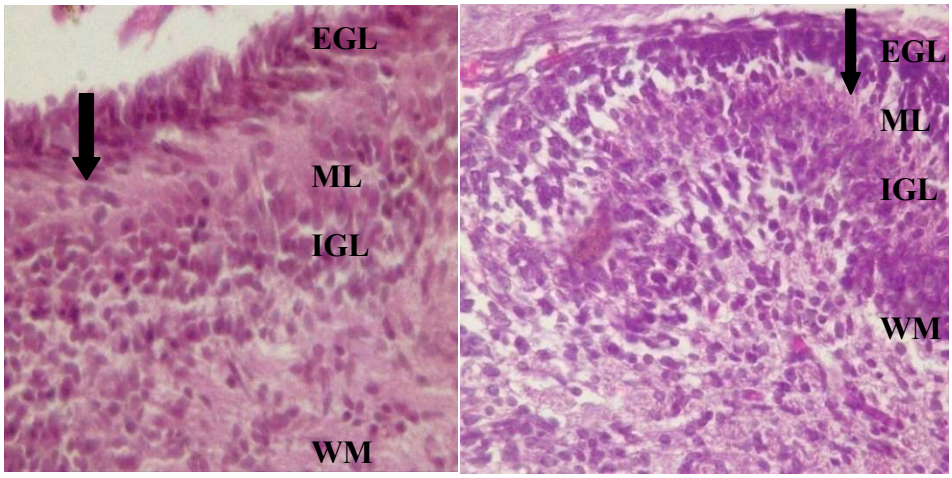
4.7.9 Pyknotic Indices Values (%)

Pyknotic indices of the cells of the EGL were obtained per 4000sqμ. More cells were dying as the pups grew. Peak values were obtained on PND 21 with group I measuring 8.5%, group II - 13.6%, group III - 12.0% and group IV - 16.6% respectively.

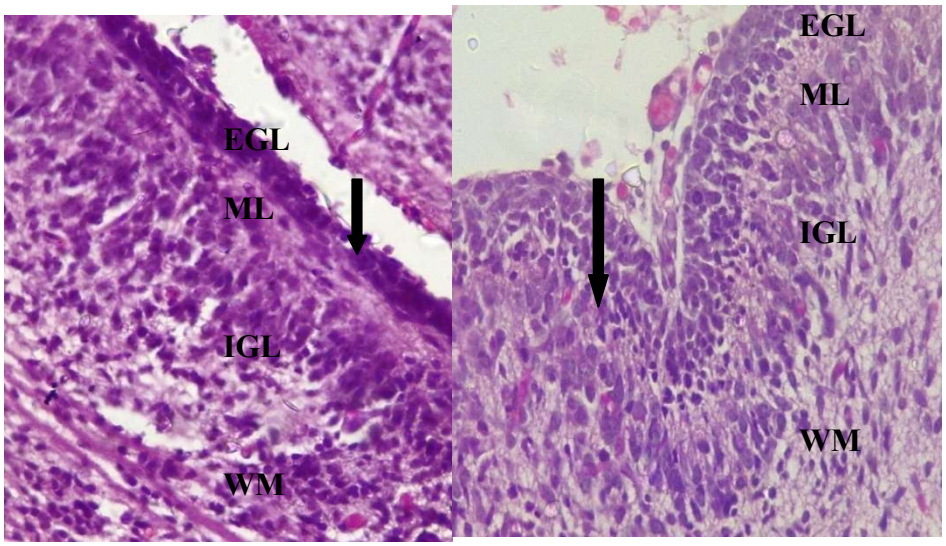
Table 4.15: Pyknotic indices on PND 7, 14, 21

	Group I n = 10	Group II n = 10	Group III n = 10	Group IV n = 10
PND 7	5.5	6.3	5.8	5.0
PND 14	5.9	4.6	6.3	9.0
PND 21	8.5	13.6	12.0	16.6

PND	Postnatal Day
N	Total number of animals used
%	Percentage of cells in pyknosis

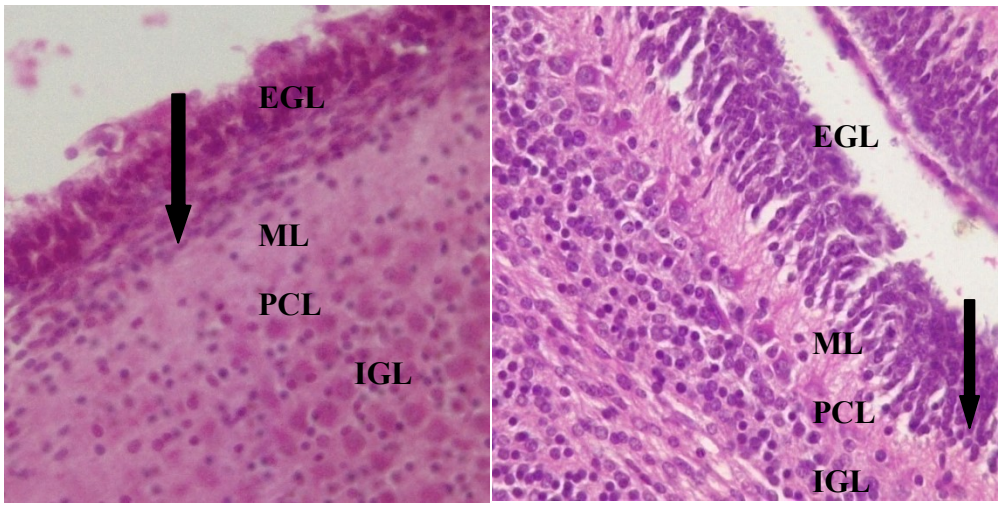


C0 1PND0



2PND0 3PND0

Plate 4.1: Photomicrographs showing the few layers of granule cells in the cerebellar cortex on PND 0 (PND – Postnatal Day; EGL- External Granular Layer; IGL- Internal Granular layer; ML – Molecular Layer; WM - White Matter; C0 – Control; 1PND0 – 100mg; 2PND0 – 200mg; 3PND0 – 300mg) → - Arrow shows the width of the EGL (H&E stain; Mag. X 40).



C7 1PND7

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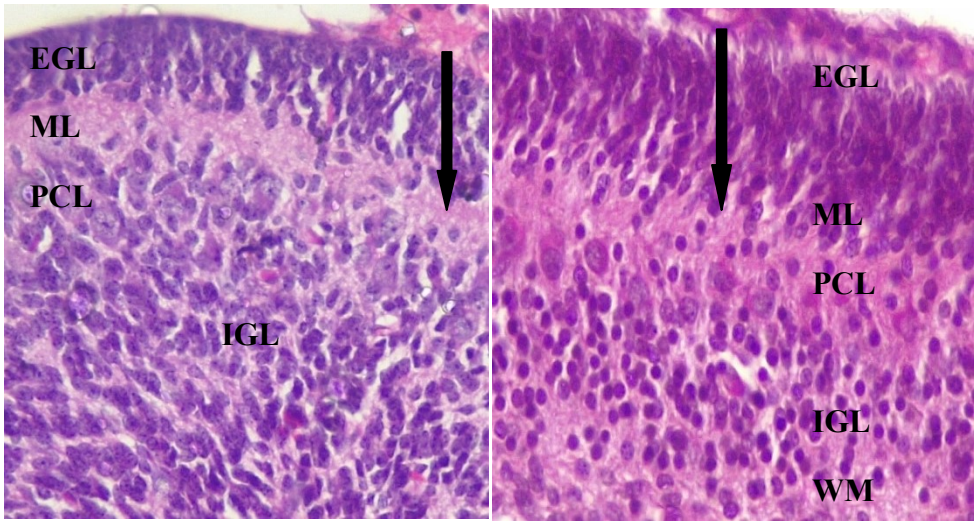
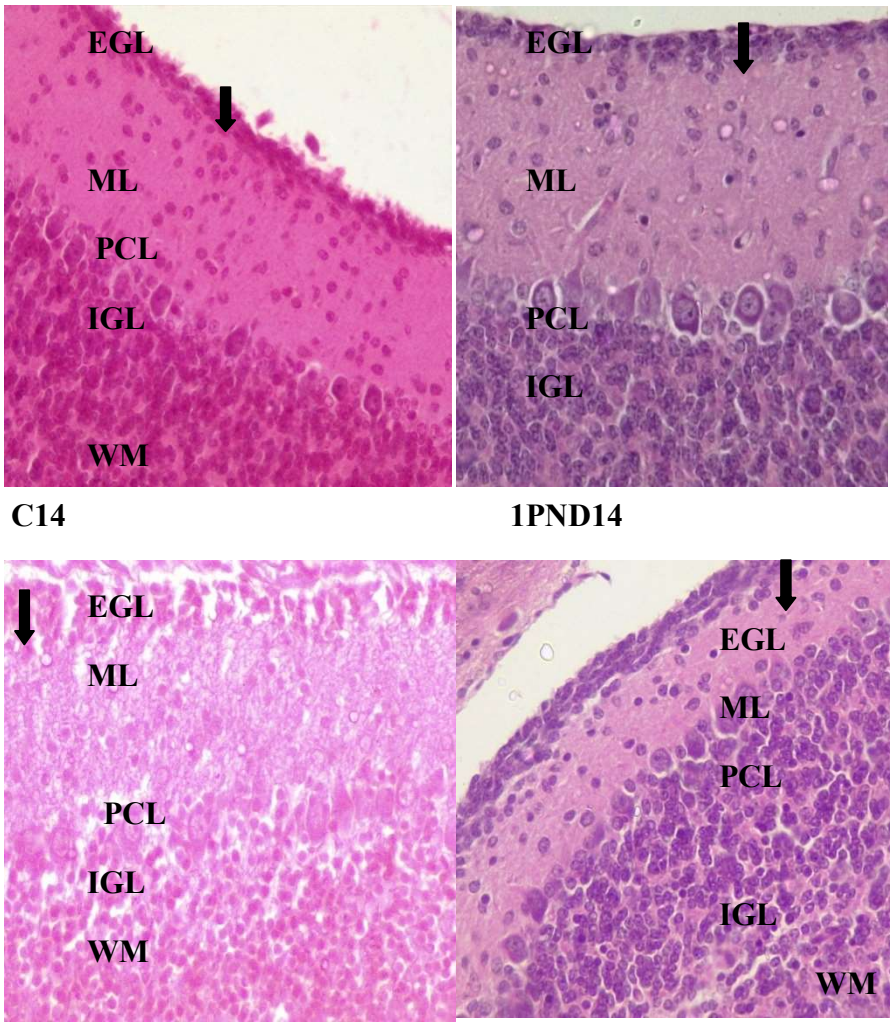


2PND7 3PND7

Plate 4.2: Photomicrographs showing the maximum layers of cells in the cerebellar cortex on PND 7 (PND – Postnatal Day; EGL- External Granular Layer; IGL- Internal Granular layer; ML – Molecular Layer; WM - White Matter; PCL - Purkinje Cell Layer; C7- Control; 1PND7 – 100mg; 2PND7 - 200mg; 3PND7 – 300mg) ➡ Arrow indicates the width of the EGL (H&E stain; Mag. X 40).



C14 **1PND14**

2PND14 **3PND14**

Plate 4.3: Photomicrographs showing the a reduced cell layer in the cerebellar cortex on PND 14 (PND- Postnatal Day; IGL- Internal Granular layer; ML – Molecular Layer; WM - White Matter; PCL - Purkinje Cell Layer; C14 –Control; 1PND14 – 100mg; 2PND14 – 200mg; 3PND14 – 300mg) ⟶ - Arrow indicates the width of the EGL (H&E stain; Mag. X 40).

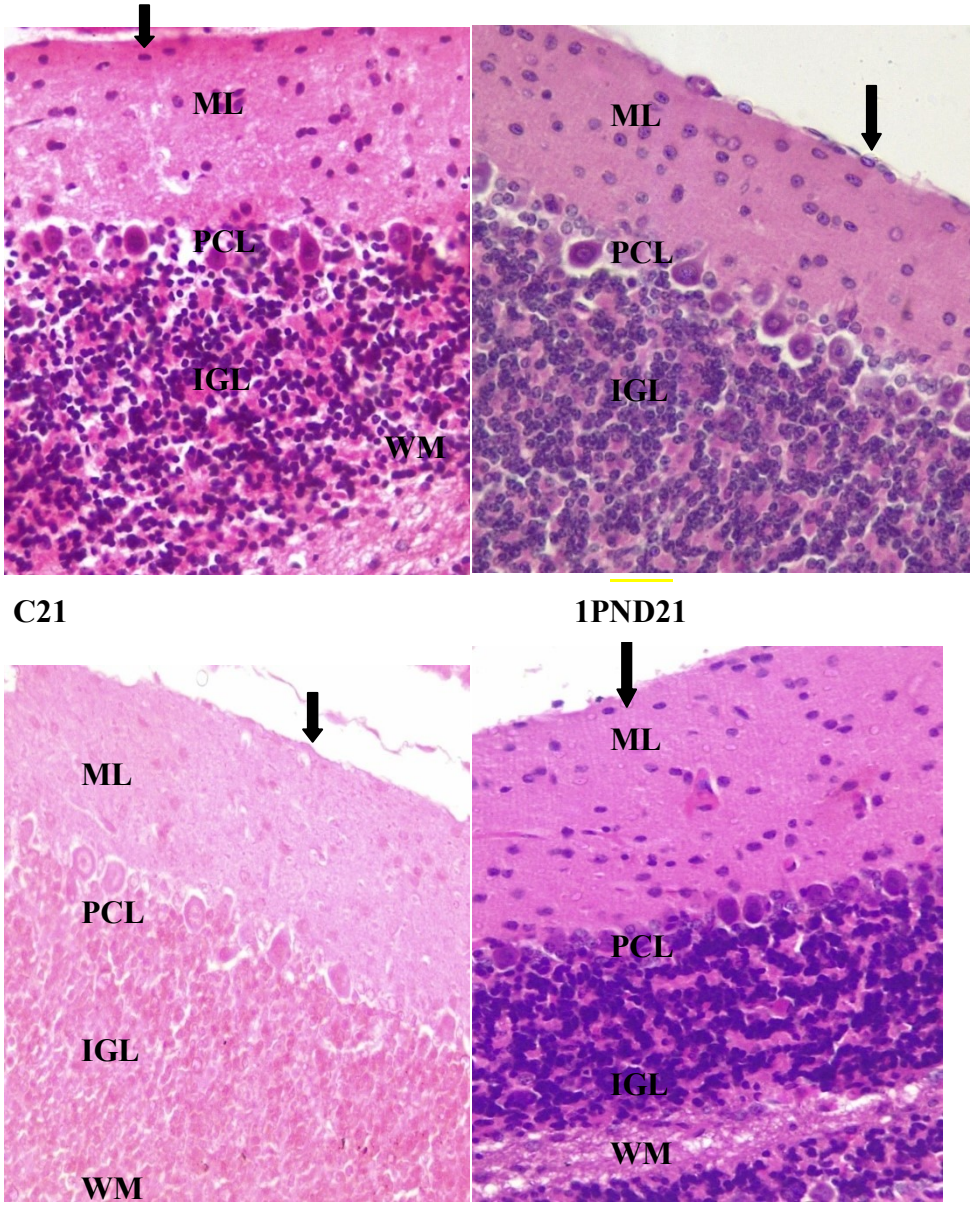


Plate 4.4 Photomicrographs showing no cell layer in the cerebellar cortex on PND 21 (PND – Postnatal Day; IGL- Internal Granular layer; ML – Molecular Layer; WM - White Matter; PCL – Purkinje Cell Layer; C21 – Control; 1PND21 – 100mg; 2PND21 – 200mg; 3PND21 – 300mg) ➡ - Arrow shows the disappearance of the EGL (H&E stain; Mag. X 40).

4.7.10 Width of the Proliferative Layer (µm)

The mean width of the proliferative layer taken on PND 7, being the day of maximum EGL thickness was compared among the groups (Table 4.11). No variation worthy of note was present in the mean width of group I (123.50 ± 27.41)µm and group II (134.50 ± 11.82)µm, group III (113.60 ± 14.32)µm and group IV (108.30 ±15.50)µm.

Table 4.16 Width (µm) of the Proliferative Layer (Mean ± SD)

N=10	Group I	Group II	Group III	Group IV
Mean	123.50	134.50	113.60	108.30
SD	27.41	11.82	14.32	15.50
SEM	8.67	3.74	4.53	4.90
P-value		0.2549	0.3247	0.1442

*P < 0.05

PND	Postnatal Day
SD	Standard Deviation
SEM	Standard Error of Mean
N	Total number of animals used

4.7.11 Width of the Postmitotic Layer (µm)

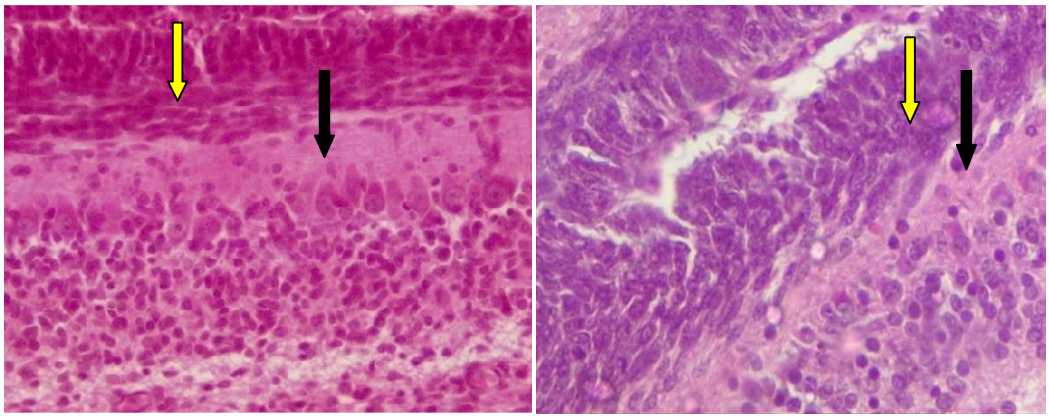
The mean width of the postmitotic layer taken on PND 7, being the day of maximum EGL thickness was compared among the groups as shown in Table 4.14. A significant difference was observed between the mean width of group I (130.60 ± 32.53)µm and group II (156.60 ± 9.96)µm, while there was no difference between group I (130.60 ± 32.53)µm and group III (134.40 ± 29.34)µm and group IV (127.80 ± 31.88)µm.

Table 4.17 Thickness (µm) of the Postmitotic Layer (Means ± SD)

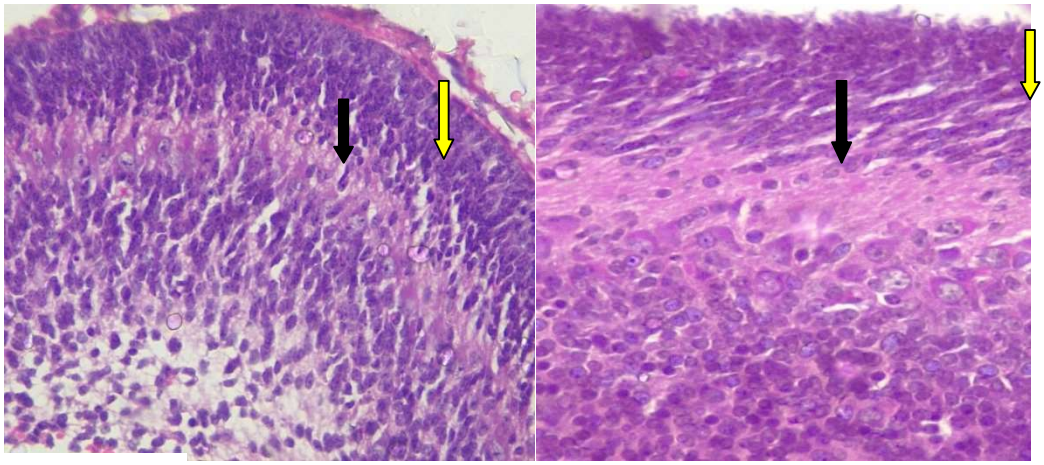
N=10	Group I	Group II	Group III	Group IV
Mean	130.60	156.60	134.40	127.80
SD	32.53	9.96	29.34	31.88
SEM	10.29	3.15	9.27	10.08
P-value		0.0265*	0.7870	0.8481

*P < 0.05

PND	Postnatal Day
SD	Standard Deviation
SEM	Standard Error Mean
N	Total number of animals used
*	P value is significant at < 0.05



C7 1PND7



2PND7 3PND7

Plate 4.5: Photomicrographs of EGL Zones on PND 7; ⟹ – Proliferative Layer, ➡ Premigratory Layer (PND – Postnatal Day; C7 – Control; 1PND7 – 100mg; 2PND7 – 200mg; 3PND7 – 300mg; EGL – External Granular layer). No difference occurred in the proliferative zone between all groups while there was a change in the premigratory zone on PND 7 (H&E; Mag. X 40).

4.7.12 Radially oriented granule cells (Cellular density per 100,000sqμ)

The mean granular cell count done on PND 7 and 14 was compared between the groups. The mean migrating cell count for group I on PND 7 and 14 (1.66 ± 0.48; 1.28 ± 0.43) was significantly lower than those of group II (2.64 ± 0.62; 2.98 ± 1.02), group III (2.93 ± 0.93; 2.30 ± 0.42) and group IV (2.70 ± 0.29; 3.08 ± 0.86) respectively.

Table 4.18a: Density of radially oriented granule cells in the cerebellar cortex of pups on PND 7 (Means ± SD)

	Group I n=5	Group II n=5	Group III n=5	Group IV n=5
Mean	1.66	2.64	2.70	2.40
SD	0.48	0.62	0.73	0.72
P-value		0.024*	0.029*	0.009**

*P < 0.05

** P < 0.01

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

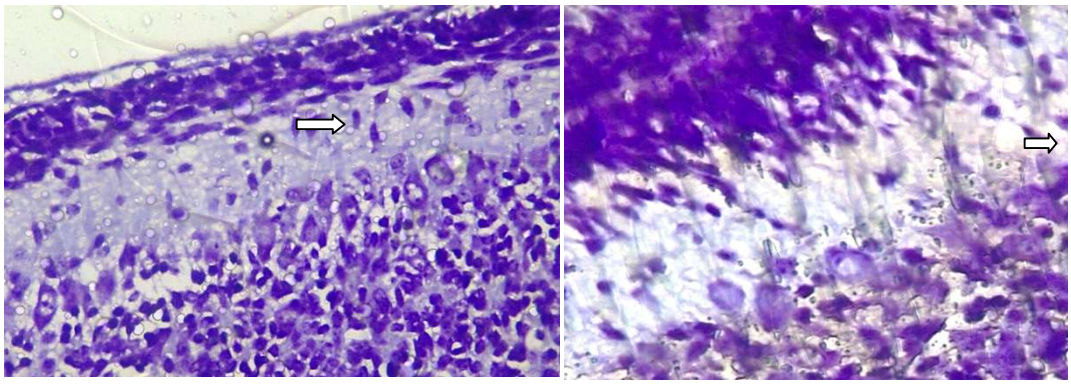
Table 4.18b: Density of radially oriented granule cells in the cerebellar cortex of pups on PND 14 (Means ± SD)

	Group I n = 5	Group II n = 5	Group III n = 5	Group IV n = 5
Mean	1.28	3.02	2.42	3.08
SD	0.43	0.89	0.44	0.86
P-value		0.004**	0.004**	0.003**

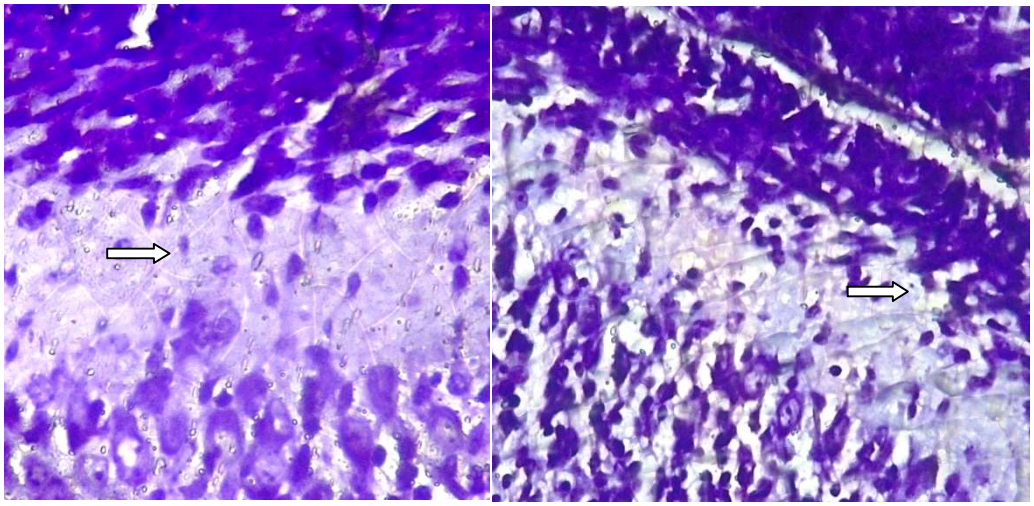
*P < 0.05

** P < 0.01

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

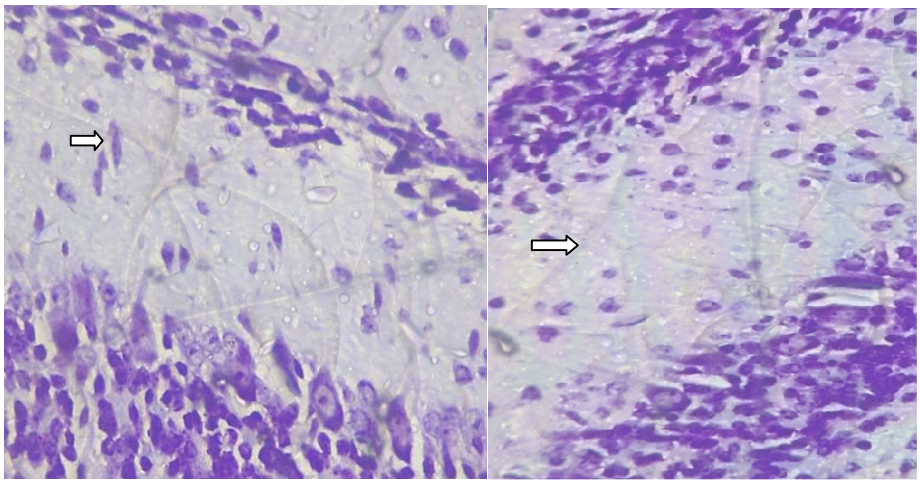


C7 **1PND7**

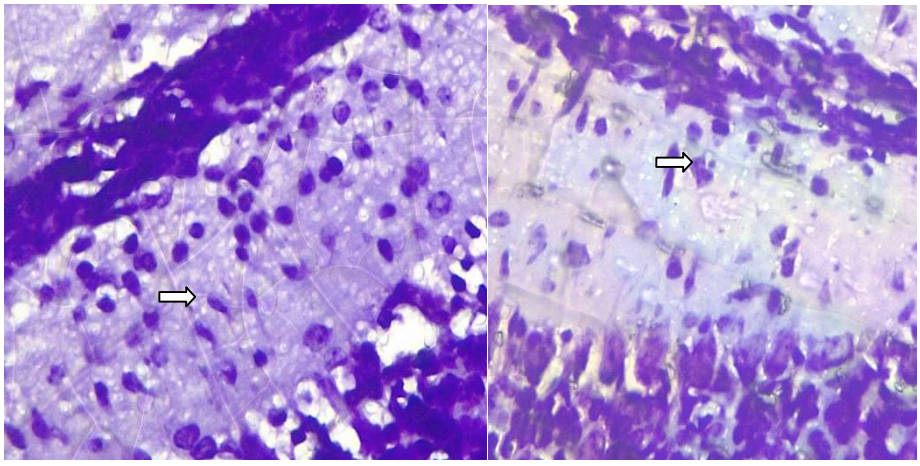


2PND7 **3PND7**

Plate 4.6: Photomicrographs showing radially oriented cells on PND 7. Radially oriented cells (PND – Postnatal Day; C7 – Control; 1PND7 – 100mg; 2PND7 – 200mg; 3PND7 – 300mg) ⇨ Arrow shows more radially migrating cells in the molecular layer of the experimental groups (Cresyl Violet Stain; Mag. X 40).



C14 **1PND14**



2PND14 **3PND14**

Plate 4.7: Photomicrographs showing radially oriented cells on PND 14 (PND - Postnatal Day; C14 – Control, 1PND14 – 100mg; 2PND14 – 200mg; 3PND7 – 300mg) ⟹ Arrow shows more radially migrating cells in the molecular layer of the experimental groups (Cresyl Violet Stain; Mag. X 40).

4.7.13 Persistence of the EGL (%)

Group I and group II recorded 20% persistence of the external granule cells on PND 21 while group III recorded 5% and group IV had no animals retaining the cells (Table 4.14).

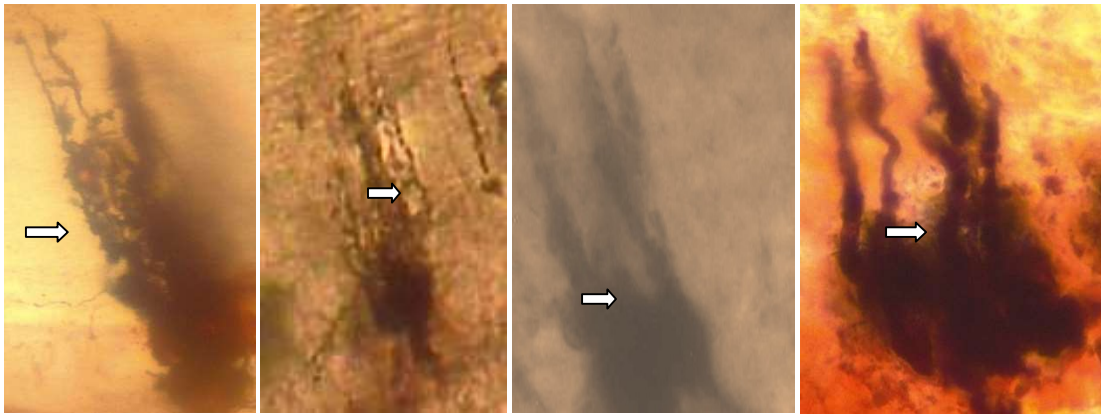
Table 4.19 Persistence of the EGL on PND 21

Groups	Animals (N=20)
Group I	20%
Group II	20%
Group III	5%
Group IV	0%

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
%	Percentage of animals who retained the EGL on postnatal day 21

4.7.14 Bergmann Glia Morphology

The morphology of Bergmann glia seen in group I showed voluminous and thinner cytoplasmic processes, increased spinous processes and longer pial directed processes of Bergmann glia. Group II showed thinner cytoplasmic processes while group III and group showed reduced cytoplasmic processes and absence of spines.



Control **1PND14** **2PND14** **3PND14**

Plate 4.8: Photomicrographs of Golgi staining of the cerebellar cortex of control and experimental mice on PND 14. Note the voluminous and thinner cytoplasmic processes, increased spinous processes and longer pial directed processes of Bergmann glia in the control mice; arrows (white) show presence or absence of spines (PND – Postnatal Day; C14 - Control; 1PND14 – 100mg; 2PND14 – 200mg; 3PND14- 300mg) Mag. X 40

4.7.15 Percentage Positivity of Proliferating Cells (%)

Proliferating cells immunostained with Ki-67 antibody. The percentage positivity was determined and compared between the groups on PND 7 and 14. On PND 7, the mean percentage positivity for group I (94.75 ± 0.0%) was lesser than group III (98.07 ± 0.81%) and was significant, while group I (94.75 ± 0.0%) was not significantly different from group II (92.56 ± 1.27%) and group IV (96.92 ± 1.01%). On PND 14, group I (95.63 ± 2.67%) had less proliferating cells than group II (99.25 ± 0.32%) and was statistically significant. No difference existed between group II (99.25 ± 0.32%) and group III (97.12 ± 1.96%) and group IV (96.13 ± 1.04%).

Table 4.20 Percentage Positivity of Proliferating Cells (%) (Means ± SD)

Groups	PND 7 n = 5			PND 14 n = 5		
	Mean	SD	P-value	Mean	SD	P-value
Group I	94.75	0.0		95.63	2.67	
Group II	92.56	1.27	0.4409	99.25	0.32	0.0166*
Group III	98.07	0.81	0.0031**	97.12	1.96	0.1658
Group VI	96.92	1.01	0.2329	96.13	1.04	0.7051

P<0.05*

** P < 0.01

Group I dams received only water while dams in groups II, III and IV received 100mg, 200mg and 300mg Tryptophan per kg body weight respectively daily.

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

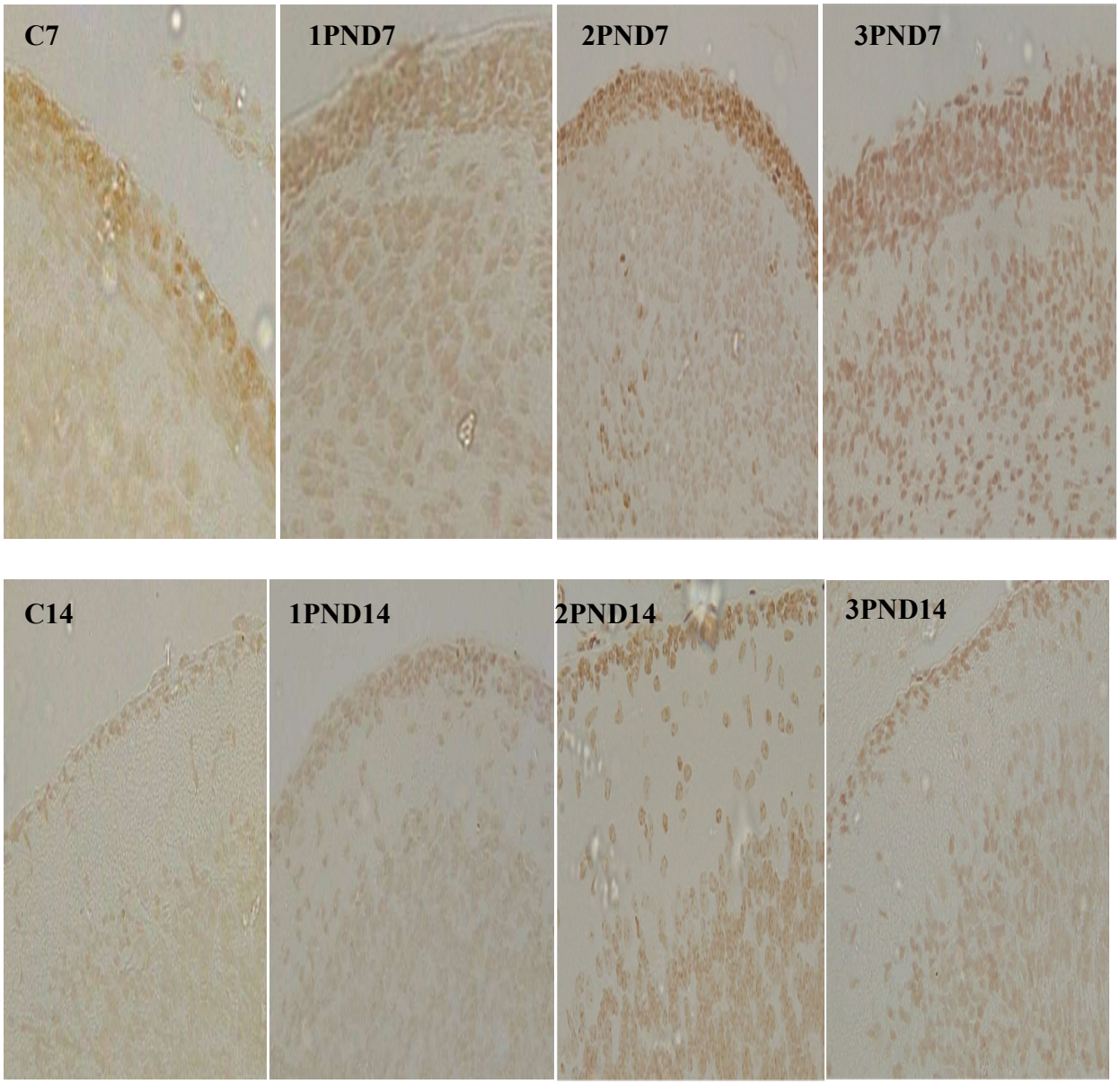


Plate 4.9: Photomicrographs of Ki-67 immunostaining showing a higher positivity of the proliferating cells in all experimental groups PND 7, 14 – (PND - Postnatal Day; Control C7, C14); PND 7, 14 – (100mg 1PND7, 1PND14); PND 7, 14 – 200mg 2PND7, 2PND14); PND 7, 14 – 300mg 3PND7, 3PND14) Mag. X 40

4.7.16 Density of Apoptotic Cells (Cellular density per 100,000sqμ)

The sum of dying granule cells was determined using Bcl-2 antibody and compared among the groups on PND 7. The mean dead cells for group I (94.74 ± 0.0) was significantly lower than group III (98.07 ± 0.81), while group I (94.74 ± 0.0) was not significantly different from group II (92.56 ± 1.27) and group IV (96.92 ± 1.01).

Table 4.21 Number of apoptotic granule cells in the molecular layer (Means ± SD)

Groups	PND 7 n = 5		
	Mean	SD	P-value
Group I	0.41	0.21	
Group II	0.30	0.09	0.441
Group III	0.12	0.13	0.003**
Group IV	0.15	0.15	0.233

*P <0.05

** P < 0.01

PND	Postnatal Day
SD	Standard Deviation
N	Total number of animals used
*	P value is significant at < 0.05

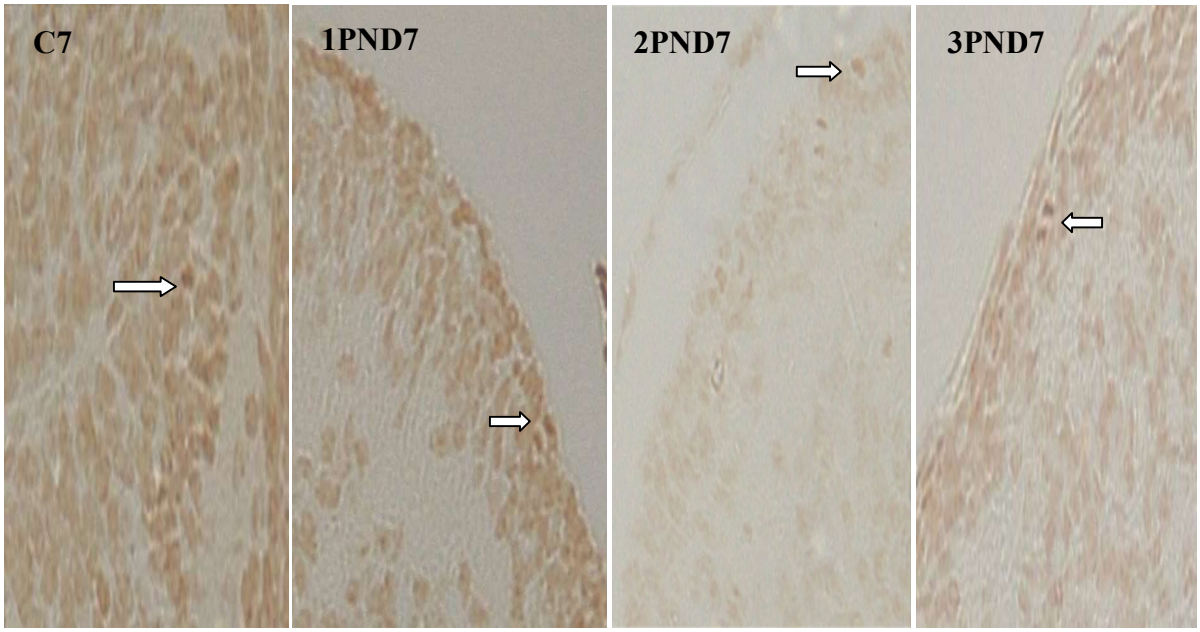


Plate 4.10: Photomicrographs of Bcl-2 immunostaining showing more apoptotic granule cells in control than others (White arrows) on PND 7 (PND – Postnatal Day; C7 – Control; 1PND7 – 100mg; 2PND7 – 200mg; 3PND7 – 300mg) Mag. X 40

CHAPTER FIVE

DISCUSSION

5.1 Overview

This study was done to examine the persistence of the EGL of mice pups following Trp administration with interest on granule cells and Bergmann glial cells. Proliferation, apoptosis of granule cells and possibility of neurogenesis in the nascent cerebellum was also investigated. The action of monoamines in neurogenesis, growth of cells and changes on the young cerebellum has been examined by Del Angel-Meza *et al*., 2001). They reported that Tryptophan deficient animals demonstrated reduced body, brain and cerebellar weights than animals who were supplemented with Tryptophan meaning that it affects the growth rate of the animals and that postnatal supplementation of Trp influences the developing CNS.

It is well accepted that serotonin with Trp as its source performs various activities in the CNS such as to slow down aggression and adjust the body's response to stress proposing that when Trp is obtained in the diet it may split as a substance that freely mops up unwanted ions that are harmful to the body and offer valuable contribution on the capacity of the organism to remove potentially damaging oxidizing agents. Nearly all the events of existence and diseased conditions are associated with the perpectives of these harmful molecules that can provoke damage in the organism when they are present in high quantity.

From this study, Tryptophan deficiency during development affects the CNS in different ways depending on the timing and other activities that may be developmental in the brain. The effect Tryptophan gives is relative to the dose. At low doses EGL persist but at high doses it speeds up the movement of the GCs inwards and consequently the disappearance of the EGL.

Tryptophan is known to have various influences in different circumstances. In pregnancy, circulating Trp availability is increased which may be an important physiological event in relation to fetal development and growth (Badawy, 1988). In patients with differently caused amenorrhea, women with anorexia nervosa recorded lower value of serum Trp while serum Trp remained the same in patients who have hypothalamic amenorrhea or hyperprolactinemia in comparison with healthy subjects. The role of Trp in mood disorders have been reported, but remains unclear the complex systems underlying Trp and nervousness. It is probable that activities of enzymes in Trp metabolism pathways perform a principal function in the

mechanisms that are molecular which underlie disorders of moods as proposed by the relationship of Trp and its metabolites (Funakoshi *et al.*, 2011). Various nutrients such as Trp and its catabolite serotonin and some enzymes in Trp metabolism such as Tryptophan hydroxylase and Trptophan-2, 3- dioxygenase have been proposed to have modulatory roles. Kanai *et al*., 2009 also reported an unwavering molecular connection between Trp metabolism and one's psychological well being.

Trp and serotonin both have parts to play in the control of diverse conductions such as mood, desire for food, cognition and sleep (Bell *et al.*, 2001). A boost in the serotonin level activity in the brain activity is proposed to improve cognitive effect, while a drop in Trp and serotonin concentrations in the brain is known to damage recalling ideas to mind (Lieben *et al*., 2004). A diet with increased quantity of tryptophan increased the production of brain serotonin and other associated behaviors and evidence reveals that Trp and serotonin perform a vital function in this activity. Increased brain serotonin shows an improved intelligence in humans and animals while reducing brain serotonin concentrations by short-term Trp exhaustion shows an impaired cognition (Khaliq *et al*., 2006). Availability of dietary precursors of neurotransmitters can affect the function of the brain. Shortly after birth, pups were commenced on breast milk. Traces of tryptophan were found in the breast milk, this has been reported to produce real sleep at night that was much longer than infants fed on formula (Cubero *et al.*, 2005). In the early days of life, the measure of Trp obtained through breast milk has a repercussion on the anabolism of melatonin and on the others in the course of the nocturnal cycle.

Reduction in protein levels decreases the speed at which cells differentiate and leads to distortions as the cells migrate and affects the formation of synapses. Additionally, a reduction of both brain serotonin concentrations and its activity on nerves occurs as brain develops in a state when Trp is lacking in the food (Del Angel-Meza *et al*., 2001).

In this study, pups had parameters that showed the effects of both high and low doses of Trp on them. The effects were noticed either in the delayed or rapid disappearance of granule cells in the third week of life.

5.2 Tryptophan and Neurogenesis

The cerebellum is a typical model to appreciate the incorporation of locally controlled varieties of nerve cells in the young CNS. Cerebellar GCs are profusely the major cells in the vertebrate

CNS and is acknowledged to perform essential roles in the passage of mossy fibers into the cerebellum (Su *et al.*, 2006).

The compounds named in adult neurogenesis are indicators of development of recently formed nerve cells which participate in the maturation of GCs in the dentate gyrus of the hippocampus. Fluoxetin is a preferred drug used in the management of depression, it is selectively used to inhibit serotonin or enhances nerve cell rebirth in the hippocampus with a double fold higher level of 5-HT from Trp than control mice (Marlberg *et al.*, 2000).

Fluoxetine inhibits a substance that transports serotonin and raises the levels of serotonin in the gaps found between synapses. This 5-HT performs an active function in the early maturation of CNS, inclusive of multiplication of cells and its maturation and is vital for appropriate connection of the brain (Gaspar *et al*., 2003). When young adults are exposed to fluoxetine, but not in fully grown-ups, there is an improvement in the rate at which nerve cells regrow in the ventral hippocampus (Klomp *et al*., 2014). Additionally, 5-HT entailed in the adult growth of cells in the hippocampus (Brezun and Daszuta, 1999). Although neurogenesis reduces as age advances, it is likely to be roused by external factors, or in conditions such as depression with a number of antidepressants (Paizanis *et al*., 2010) including fluoxetine, which aids multiplication of cells in juvenile rodents (Kodama *et al*., 2004) but not in advanced rats (Marlatt *et al*., 2013).

Although, adult neurogenesis is frequently used to quantify changes in the structure in vertebrates (Lucassen, 2010), it is widespread in the phase following birth and decreases with age (Heine *et al.*, 2004).

5.3 Total Serum and Brain Tryptophan

Total serum protein was compared among the groups. The total serum level for group II was considerably elevated than group I and group IV on day 21. Pups in group III did not demonstrate any disparity in comparison with group I but group IV was very much lower than group I. Trp is available in the serum, attached partially to albumin and circulates freely. Circulating Trp that did not bind crosses the hematoencephalic barrier and is changed into 5-HT in the brain by the activities stimulated by enzymes of decarboxylation and hydroxylation (Comai *et al.*, 2010).

In mice with Trp deficiency, the Trp concentration in the serum and liver were strikingly increased by tryptophan dioxygenasedistruption, and high Trp level in plasma enters the brain using a protein unit carrier at the blood brain barrier thereby enhancing the volume of Trp in the brain. This is expectedly going to be the reason for the increased level of Trp seen in group II animals with minimal dose of the amino acid. Afterwards, increased Trp levels in the brain are converted to serotonin by TPH and later to 5-Hydroxyindole acetic acid. This is may be responsible for the traces found in the serum of animals given higher doses of Trp in group III and IV because a lift in blood Trp will raise its entry into the brain (Fernstrom, 2013).

Vieing for entry through the BBB, LNAA gains access through (Peters, 1991). The rise in the concentrations of plasma Trp, without the other LNAA, and the resulting boost in the Trp/LNAA proportion in mice with Trp deficiency proposed an increment in Trp transfer across the BBB. HPLC analysis indicated a concentration of Trp in the brain was exceedingly higher in Tryptophan deficient micethan in the wildtype (Funakoshi *et al*., 2011).

Conversely, in a related study undertaken by De Marte *et al*., 1985, mice that were fed on low quantity of Trp, protein deficient and control diets for a prolonged period of about 78 weeks showed that plasma Trp level was diminished by both types of restrictions in the diet. The proportion of 5-HT was reduced in animals on Trp restriction diet probably due to possession of more LNAA which struggle with Trp for entry into the brain. This indicates that the less the quantity of Trp in the diet overtime with a higher proportion of LNAA the lesser the level of Trp obtained in the brain, it is also probable that a long period of exposure to the deficient diet and quantity of plasma Trp matters. It has been proposed that lack of Trp impairs growth and maturation of the CNS by diminishing the levels of 5-HT. These findings opined that lack of Trp in nutrition affects development and aging by affecting CNS synthesis of protein or 5-HT metabolism (Segall and Timiras, 1976).

Brain Trp concentration is dependent on the serum concentration of a set of neutral protein units, which participate alongside brain Trp utilisation. Irregularities in brain Trp concentrations after food intake may be as a result of this process (Fernstrom & Wurtman, 1972) seeing brain Trp level is not the same as free plasma Trp (Madras *et al*., 1974) and are controlled by its ratio to LNAA (Trp:LNAA). Plasma tryptophan proportion therefore determines brain typtophan entry and 5-HT production in rats (Fernstrom *et al.*, 2013).

5.4 Morphometrics

5.4.1 Brain and Cerebellar Weight

Pup brain weight in the group I was significantly lesser than group II; group III also measured less than group I on day 7. However, the brain weight of pups in group IV was consistently lower compared with the controls. The optimal dose for effectiveness seems to be 100mg. Trp has a strong involvement in the activity of growth hormone (Rivest, 1991); and in the developing cerebellum it influences proliferation of glioblast, production of young neurons and the migration of cells (Bishop *et al.*, 1985). Linked with this are the effects seen when an optimal dose was administered especially as seen in group II animals and comparing them with group I rats.

Also, weight and 5-HT levels in whole-blood revealed a strong negative correlation (Albay III *et al.*, 2009). The occurrence of many neonates with reduced weight but no visible anatomical defects influenced this relationship (Janušonis *et al*., 2006). Very low levels of maternal peripheral serotonin lead to mouse embryos that are very small and display defects in several body systems (Fligny *et al.*, 2008). It is nevertheless, speculated that a reduction in the mother's serotonin could lead to decreased body weight and excess production of peripheral serotonin to be compensated for in the offspring. When peripheral 5-HT precursor 5-hydroxytryptophan (5-HTP) was administered to rats that were pregnant, this result in embryos with low body weight (Salas *et al.*, 2007). Presence of Trp in the brain may have effects on the growth of the brain during the production of serotonin since tryptophan can cross the BBB (Hoerder-Suabedissen *et al.*, 2008).

Bucci *et al.*, 1982 seem to confirm the hypothesis put forward by some investigators, according to which the derangement of Trp metabolism which follows a Portocaval shunt (PCS) may perform a vital function in patients who have hepatic encephalopathy (Cummings *et al*., 1976). Such a derangement causes only mild toxic effects, characterized mainly by weight loss and brain changes (Doyle, 1967; Cavanagh and Kyu, 1971), not detectable by behavior observation (Wasterlain *et al.*, 1978). The observations of Bloxan *et al* (1977) have shown that toxic effects and behavioural changes occur when Trp metabolism is affected when the animals were given more of this amino acid. This may be due to individual free essential amino acids been normally present in the body in a relatively fixed ratio (Iob *et al.*, 1970), whenever such a ratio is altered,

untoward effects may occur by different mechanisms. One of these may be a direct toxic effect exerted by the higher concentration of one of the amino acids (Iob *et al.*, 1970), Trp in this case, the high quantities of this amino acid which reach the liver from the intestine and are mainly metabolized through the kynurenine pathway (Knox and Greengard, 1964). Using a quantity of 200 mg/kg of L-Trp and higher, we may assume that L-Trp is not only toxic to the liver parenchyma (Bucci and Chiavarelli, 1979) but also to the brain of PCS rats. This is similar to our finding that at a much higher dose of 300mg/ kg of L-Trp the animals recorded lower body and brain weight.

The cerebellar weight of group I weighed less than group II on day 7 and 14, while group III also weighed less. The effective dose for Trp usage seems to be 100mg in which case, both brain and cerebellar weight were at their peak as corroborated by Khaliq *et al*., 2006. However, group IV recorded lower weights all through the experiment. The lower weight recorded by group IV might be due to the toxic effect Trp has at higher doses on both the liver and brain which results in reduced body and brain weights (Del Angel-Meza *et al*., 1989).

In a related study, changes occurred after birth in brain and body weights in IGF-II/ IGF-I genetically altered mice and normal controls. Growth of the animals was not unusual followed by a swift gain in brain weight between PND 7 and 28, accompanied by a steady rise to PND 130. Growth of the brain in the genetically altered mice was considerably enhanced. On PND 7, there was no change in the weight of the brain between the two groups, rather a boost in size was observed in genetically altered mice on PND 14 (O'kusky, 2000).

5.4.2 Cerebellar Cortical Width

In rodents, the size of the GM in the cerebellum is roughly 3.8-fold compared to its WM (Bush and Allman, 2003), using results obtained in the CC (Zhang and Sejnowski, 2000), in mouse, the proportion is about 4.5- fold. The CC is the focal point of intense research because of the presumption that it is in charge of regulating locomotion and adjusting to conditions that are special, and recently, it is concerned with accumulating memories for different periods (Attwell *et al.,* 2002). In the cerebellar cortex, many structural changes that depend on age take place which have been extensively investigated in rodents, *Macaca nemestrinas,* gerbils and human beings, (Jernigan *et al.,* 2001). The outcome from these experiments revealed a drop in the width of the CC, death of neurons, hyperplasia and hypertrophy of the astrocytes in elderly individuals (Zhang *et al.*, 2006).

However, in this study, the cerebellar cortical width for both the group I and experimental animals did not show any difference in width. It is probable that Trp does not necessarily affect the expansion of the cerebellar cortical layer in the preliminary three weeks after delivery. The manner of growth observed for the width of the ML in all the regions of the cerebellum are about the same, the width of the IGL in all the parts studied are also about the same. The EGL and the ML have consistent widths throughout the CC, but the width of the IGL increases randomly; as the cerebellum folds (Raaf and Kernohan, 1944). All these may be accountable for the changes not seen in the width of the CC in all the groups and considering their nascent age too. The EGL and ML have consistent widths throughout the CC, but the thickness of the IGL increased due to cells that migrated from the EGL. Cells in the IGL are packed compactly such that there is a union of the soma, allowing an outstandingly undersized mass of neuropil to divide the cells. The dendrites at the top of these GCs move into the ML to accept the infinite greater part of the contacts between synapses from afferent axons (O'kusky *et al.*, 2000).

A major diminution in the width of the ML will result in a drop in the full width of the cerebellar cortex. Decline in width in the ML may be as a result of the gradual reduced foliation of aged PCs dendrites (Hadj-Sahraoui *et al.,* 2001), while expansion in the width of the IGL is for the most part due to cellular proliferation. The precise significance of the reduction in the thickness of cerebellar cortex is not fully understood, while a decrease in the quantity of neurons leads to general motor behavior deficit which is for the most part established (Caston *et al.,* 2003).

5.5 External Granular Layer

The mice cerebellum is very immature at birth and major histogenetic events take place postnatally, from the first to the third week. The EGL is a short-lived group of proliferating cells which is positioned directly below the pia of the cerebellum of infantile animals with backbone.

Just after delivery, the soma of the EGL cells steadily move down on the BG to become GCs in the IGL, with the parallel fiber axons lenghtening in the ML producing the typical T-shape morphology as shown in the in vitro configuration of the T-shaped GC (Yamasaki *et al.*, 2001).

These examinations demonstrate that the ability to migrate is innate in these cells and the T-shape structure are similar elements observed in stem cells *in utero* that will change into the prospective granule cells.

On the PND 2, the EGL is before now well defined, within the first week after delivery, its capacity is increased. Afterward, the growth curve is unhurried and as the third week approaches to an end, the layer disappears. EGL cells in rat cerebellum appear initially in the germinal trigone in the few days to the expiry of embryogenesis (Altman and Bayer, 1978), it penetrate the cerebellum immediately after delivery and multiply there. Shortly after delivery, cells start to travel from the EGL, majorly to the IGL to become GCs (Altman, 1972).

The EGL is a temporal structure which disappears after production of the several varieties of nerve cells present in the CC. Researchers have postulated various days for the disappearane of the EGLFujita, 1967 reported that the whole quantity of the internal GCs appears post-natally in the EGL between PND 1 and PND 18. The EGL vanishes by PND 20 in the wildtype mouse (Shimada, 1966). Altman, 1971, also reported that the EGL is present over the cerebellar cortex until about 21 days of life. In this present study, the EGL disappeared on day 21 in agreement with Altman, 1971. However, about 20% of the animals in the group I and group II retained 20% of the GCs, 5% in group III retained the cells and 0% retained the cells in group IV. This showed that increasing levels of Trp elevated the momentum of the movement of these cells into the IGL. Trp at lower doses was able to make the granule cells persist longer while at relatively higher doses it speeded up the granule cellular movement into the IGL. EGL was persistent in group I and group II on PND 21, this may result from a possible overproduction of granule cells or when cell death and migration is slowed down. Del Angel-Meza *et al.,* 2001 reported that on PND 21, cells of the EGL were still present in the Trp deficient group whereas in the Trp fed groups, granule cell movement was absolute. Undernutrition is a factor that results in a slow disppearance of the EGL (Del Angel-Meza *et al.,* 1984).

In this study, the EGL width increased and peaked on day 7 in all the groups and then the growth slowed down, this agrees with the observation of Mares et al., 1970 who stated that the EGL is best developed on the 7^{th} postnatal day. On the 14^{th} postnatal day, group I and IV measured less than group II and III inferring that the administration of Trp to the animals has the ability to speed up the movement of granule cells and later disappear at the end of the PND 21.

According to Griffin *et al.*, 1977, the extent of the EGL indicated that animals who were administered less quantity of Trp to will lead to a smaller area on PND 8 and 11 but on PND 14 the EGL length was larger when put side by side with with animals that had higher doses of Trp. At all the three stages encountered, changes could be detected in the arrangement of cells

between the mitotic and migratory layers of the EGL. Cell production is slowed down between PND 4 and 8 which is indicated as the most sensitive period to Trp deficiency when it is likely that less Trp has a huge but a sluggish division of cells in the EGL in the early growth period. Most likely, however, adjustment occurs in the EGL which allows a part recovery of growth of the cerebellum as the days advance (Griffin *et al.*, 1977).

Moreso, the width of the EGL enlarged by four-fold from PND 0 to PND 7 in normal rats, the bulk of the EGL is produced in the first week after birth. Secondly, in early postnatal development, radiosensitive mitotic precursors are commonly situated in the EGL, the quantity of premigratory cells of the inner layer of EGL is on the increase until a maximum is reached on PND 8 – 9 (Altman, 1972).

The rate at which the width of the EGL decreases in humans is not different significantly among the cerebellar regions studied and the EGL stays until the child is about 2 years of age and there is uniformity in all the areas of the cerebellum. The growth of the EGL slows down from the fifth month *in utero* until just before the baby is delivered, but during the first 9 to 10 months after delivery, the reduction in width is very fast (Raaf and Kernohan, 1944).

In the cat, the first month is taken up with cell proliferation; the second postnatal month is concerned with cell differentiation. It is at this point that the change in zonal composition of the EGL occurs. Prior to 30 days, round cells in the external section of the EGL predominate largely over the spindle-shaped cells found at its borders. Beyond 30 days, however, proportionally more fusiform cells were found in the EGL. The occurrence of an enormous quantity of straight, fusiform cells in the ML is proportional to the dissolution of the EGL. Presumably, these spindle-shaped cells are the indifferent cells migrating towards the IGL. Similarly, the packing density and the width of the IGL are continuing to rise; the packing density increases sharply after 40 days, some 10 days following the former transformation. Since these observations are in agreement with those of Altman (1971) we may conclude that "migration of cells produced copiously in the subpial, EGL determines the growth of the cerebellar cortex" (Dacey and Wallace, 1974).

Till the second week of life in rodents, EGL contains outer and inner cellular zones: the proliferative (premigratory) and the postmitotic (postmigratory) zones. The exterior region is made up of larger epitheloid cells composed of a less dense basophilic nucleus which contains many nucleoli. The cytoplasm has a smaller capacity. The inner zone is formed by smaller cells

and are more often than not oval in shape with the longer axis directed to the middle of the cerebellum.

The distinction of both zones of the EGL and the widespread appearance of the structures that just divided and nerve cells producing DNA in the exterior region are well corroborated (Fujita *et al.*, 1966). No changes occurred in group I and the other experimental groups when the two zones were measured separately on the PND 7. This might be due to the increased aging of the animals and the proportion of the fusiform cells to the round cells which increased progressively. Nonetheless, this did not have any visible effect on manner with which the cells proliferate**.** These findings imply that there is no fixed connection between the outlook of the cells of the EGL and their competence to divide (Koppel and Lewis, 1982) and there is no difference in the functions of the discrete regions in the EGL.

EGL cells are particularly exclusive in their incessant ability to divide time and again, which goes on even after the animal is born.

There are a few factors that influence the proliferative activities within the EGL, one of them is Math1+ cells in the EGL anlage which tirelessly produce Ki67 at an elevated rate on PND 9 and after PND 11; while when the same Math1+ cells were induced by BMP4 (Mizuseki *et al.*, 2003) the cells did not stain positive for Ki67, treating the same Math1+ cells with retinoic acid resulted in a diminished Ki67 expression.

Other substances have been identified to have a negative effect on the EGL. One of such is methotrexate, a folate analogue and a folate antagonist which has been used in the management of leukemia and lymphoma (Ogungbenro *et al.*, 2014). They stated that EGL thickness of the experimental group declined 48 and 72 hours after treatment likewise the dimension of the cerebrum and cerebellum were decreased when put side by side with the control animals that were treated with saline (Sugiyama *et al*., 2015). It may be that the decline in the thickness of the EGL in the experimental group was due to a hinderance in how the external GCs that resulted from apoptosis are formed and also a cut down in cell division. Sugiyama *et al.*, 2015 concluded that when methotrexate was administered on PND 6 brought about a setback, reducing the movement of the GC from the EGL to the IGL. In a related study, 2-Gy irradiation on PND 6 produced an interruption in the movement of the GCs from the EGL to the IGL in the cerebellum of mice (Hyodo-Taguchi *et al.*, 1988). A thinning of the ML was also reported which may be the

result of partial growth of the parallel fibers and spiny dendrites of the PCs, which are the main elements of the ML (Heinsen and Heinsen, 1983). It was concluded that methotrexate on PND 6 stimulated apoptosis of GCs in the EGL and impaired their multiplication, leading to a setback in the movement of the cells and thereby causing hypoplasia of the cerebellum. However, in this study, there were positive differences between the EGL, ML and the IGL.

5.6 Migration

At the end of the last cell division, cerebellar GCs linger in the EGL between 1-2 days before starting their radial transit in the ML, however, the essence of this dormant period is not clear enough (Komuro *et al*., 2001). Extensive studies have been done on the point in time at which these cells are formed and the migratory route of new granule cell neurons (Altman, 1982). From all indications GCs are derived at the uRL, the cells extend as a slim sheet of neuron cells on the exterior of the maturing cerebellar anlage and proliferate after birth (Miale and Sidman, 1961).

In this study, Trp enhanced GC transit from the EGL into the IGL in all the experimental groups in a dose dependent fashion. These cells were flattened and radially oriented in the ML, migrating from the EGL to the IGL. Jaarsma *et al.*, 2014 investigated the function of Bicaudal-D2 (dynein adaptor proteins) in the migration of radial cerebellar GC, and reported the function of the protein in Bicd2 knockout mice as the distruption of the laminar organization of the cerebellum, indicative of distrupted radial neuronal movement. In conjunction with a distrupted inward radial migration, the ML of PND 5 - PND 15 cerebellar cortex of Bicd2 knockout mice, showed absence of granule cells with radially oriented elongated shape which distinguishes the cells for migration. The absence of spindle–shaped cells that migrate with, the clustering of GCs in the ML and the lack of an IGL indicate that inward movement of GC is interrupted to a large degree in Bicd2 knockout animals. However, the wildtype has multiple flattened radially oriented cells in the nascent ML and an intact IGL.

The GABAergic interneurons of the nascent CC are the dividing cells that reside in the IGL after birth. On delivery, SCs and the BCs start are produced and start moving to the ML (Miale and Sidman, 1961). It is assumed that malfunction that occurs as the cells migrate takes responsibility for mutants having higher cells proliferating than those in transit. The migratory discrepancies of a part of these interneurons slightly reflects less than the quantity of cells in total. It is possible that a major event that took place was that the quantity of the granule cells that have divided and

those that have migrated in the IGL went through a belated apoptosis in such a way that is more than what was obtained in the controls.

In the ML, proliferating cell density was on the rise in normal mice, but, apoptotic cell density was higher too, this indicated that as the mice advanced in age and had more cells dividing they also had more cells dying (Cocito *et al.*, 2006).

In the mutant mice, migration of the GCs is impaired, this leads to higher values of total cell density than in control mice. It is probable that a rigorously altered multiplication of cells, to a certain extent than the insufficient migration of GCs, was accountable for the changes observed in the EGL while the impairment of GC migration in the mutants was not allowed in the EGL by the Reelin deficiency and did not have higher proliferating cell values (Cocito *et al*., 2006).

Some factors regulating GC transit include mutations in *Cdk5*, *Pax6* and *Pafah1b1*ingene targeted mouse models which may be responsible for cell-autonomous GC migration anomaly (Yamasaki *et al.*, 2001). Alterations in astrotactin and neuregulin (Adams *et al*., 2002) also influence GC- BG relations and the passage of these cells.

As seen in this study and others, migration at a tangent of the GCs in the EGL may permit some activities during the process of development and the accuracy of their position along the parasagittal segments of the young cerebellar cortex.

5.7 Mitotic Cells

Very importantly, the disparity in the multiplying cells and dying cells in the layers of the young cortex is crucial to appreciating fully the magnitude of cell multiplication and programmed cell death in the development of the full-grown cerebellum in vertebrates (Cocito *et al.,* 2006). Cells that have undergone division were seen scattered at all levels in the EGL, at every period from when the animal was given birth to up to 21 days after delivery (at which time the EGL has disappeared or about to). Cells that have divided were mostly apparent in the zone close to the pia of the EGL, but were commoner in the midst of the fusiform cells in the lower portion. However, at all ages studied, few actively dividing cells are in the profound regions of the EGL, as Cajal (1911) and Mares *et al.,* (1970) reported before. Other studies have shown the presence of multiplying cells at the dorsum of the outer edge of the pia of the larval cerebellar plate of

teleostean cerebellum plus the dividing cells on the underneath part of the cerebellar zone and that cells that differentiate and migrate are inserted among them (Wulliman et al., 2011).

This study revealed that the highest mitotic index was obtained on PND 7 in agreement with Mares *et al.*, 1970 and group IV consistently had the highest values. Generally, cell proliferation as measured by the proliferating cell density of GCs was elevated in the EGL of *reln*+/+ mice (Cocito, *et al*., 2006). This implies that Trp at higher doses may be used as a mitogenic agent.

5.8 Pyknotic Cells

More cells were seen dying in all the groups on postnatal day 21 with group IV having the highest values, this could be as a result of granule cells especially as the EGL disappears after fulfilling its part in cell production. It is a possibility that the more cells divide the more cell death will likely occur. Various agents have been documented to cause pyknosis in the brain, this include ischemia, radiation among others. Dying cells assembly in the cerebellar cortex of young rats who received 200r of radiation between 4 to 12 hours, this agrees with the initial report of Hicks *et al.*, 1961 who stated similar findings when rat embryos were given exposure to the equal dose of irradiation in the CNS and embryonic rat retinae.

Loss of GCs in the EGL has been examined in the wildtype, weaver heterozygous, and weaver homozygous cerebella, with the totality of dead cells in the weaver homozygous mice increasing dramatically in relation to the quantity of dead cells seen in the wildtype EGL (Smeyne and Goldowitz, 1989). The rate at which GCs clears from the EGL of the weaver homozygous mouse shows that pyknotic cells are eliminated from the location within 4-7 hours. Estimates of cell clearing of dying cells in retina and spinal cord is consistent with the figures obtained in the EGL (Wong and Hughes, 1987).

EGL cells that are pyknotic have an unusually clumped hetero-chromatin indicative of nuclear disintegration of the cells (Cunningham *et al*., 1982) and this is the feature of the young pyknotic cycling cells (Wyllie *et al.*, 1980). Again, NOND is assumed to be a built-in cell process that may be apparent as a cell commiting suicide (Wyllie *et al.*, 1980).

5.9 Bergmann Glia

Bergmann glia are associated with the enlargement and bending of the cerebellum. A common mechanism of BG production is implicated in the development of ridges and grooves of the cerebellum (Heung *et al.*, 2017). Heterotypic gliophilic interrelations with BG processes that are

oriented in the vertical plane may guide the tangential migration of GCs. This was proposed in the embryonic cerebrum for the tangentially transiting cortical nerve cells (O'Rourke *et al.*, 1997).

A closely knit interaction amidst GC migration and BG glia maturation and is already established (Hatten and Mason, 1990). In typical rats, the straight processes of BG reduces steadily from PND 8 –12, there is an increment in the cytoplasmic excrescences (Shiga *et al*., 1983), there is a disappearance of the basal filopodia between PND 15 and 19 (Das, 1976). Bergmann glia of group I and group II animals revealed an unusual excessiveness of cytoplasmic processes in fibers which are pial-directed, which are more in number than in the group III and IV. Additionally, Bergmann glial cells in group I and group II animals indicated voluminous cytoplasmic processes, increased spinous processes, thinner cytoplasmic processes, longer pial-directed processes in contrast to the shorter pial-directed fibers reported by Del Angel- Meza *et al.*, 2001) while group III and IV showed reduced cytoplasmic processes, no spines, thicker cytoplasmic processes and shorter pial-directed processes on PND 14.

Glioblast maturation is affected by accessibility to serotonin (Lauder *et al.,* 1983), and it is known that undernutrition results in a slow disappearance of the EGL (Del Angel-Meza *et al.*, 1984), the presence of Trp availability may be responsible for the closely knit link between the effects seen in BG and the EGL migration.

Factors that influence BG include undernutrition as shown by the quantity of cells in the Nissl preparations of the CC of a PND 35 undernourished rats which indicated that inadequate feeding results in a decline in the quantity of BG (Clos *et al.*, 1977). Also, hyperthyroidism early in life influences BG in a way parallel to undernutrition. The similarity between the effects of food deprivation and hyperthyroidism on BG supports an early transient speeding up of their maturation in underfed animals resulting to a diminished quantity (Clos *et al*., 1980).

Perez-Torreno *et al.*, 1996 reported a setback in the maturation of BG in the young cerebellum in animals prenatally exposed to ethanol. The quantity of fibers per unit length was reduced in these animals proposing that altered BG maturation may result in a movement of cells that is slowed down and reduction in the number of cerebellar GCs following exposure prenatally to ethanol. They further reported that daily exposure to ethanol in utero on day 17 to 19, at a time when the

production of BG begins, altered BG postnatal growth is apparent by the decline in cell body size, number of processes and width, and increase in the length of the fibers before PND 12.

As soon as the animal is born, the BG radial fibers aid the inward transit of the maturing GCP starting in the EGL to form the IGL (Xu *et al.*, 2013). As the cerebellum of vertebrates advance in age, BG sustain organizational framework and synaptic communications (Yamada and Watanabe, 2002), and is being recommended as cells with the capacity to develop into specialized cell types in the body (Koirala and Corfas, 2010).

Bergmann glial cells take part in an indispensable function of orchestrating the bending of the CC by synchronizing the inward movement of GCP with PCL and the pia. It is a possibility that BG produces the sticking together of pial basement membranes and provides the tension that is needed for the usual folding of the CC as proposed before (Ma *et al*., 2012). On the other hand, multiplication of BG may augment the number of radial units, which invariably controls the spreading out of the surface of the cerebellum. To support this, it was observed that BG also undergo rigorous cell division till PND 15, and they exhibited plenteous proliferation in the crowns than at the bottom of the fissures (Li *et al.*, 2014).

In genetically altered mice, damaged formation of Bergmann fibers is evident shortly after PND 14, when the GCs move from the EGL to the IGL is almost done. On the other hand, other BG may balance the improper configuration of BG fibers by spreading fresh processes in the direction of the exterior of the cerebellum (Komine *et al*., 2007).

Using TUNEL and immunostaining, BG that are irregularly placed in the EGL or ML experience apoptosis, resulting in a reduction in the quantity of BG later. Besides, BG interrelate intimately with PCs as the CC develops (Yamada *et al*., 2000). It looks as though the shortage of particles available in the PCL for abnormally placed BG results to apoptosis (Komine *et al.*, 2007).

5.10 Ki-67 Proliferating Cells

Ki-67 studies demonstrated cell proliferation in the cerebellar cortex. These proliferating cells will migrate, differentiate and maturate to form cells of the cortex. As early as PND 0, cells proliferate actively within the EGL (Fujita, 1967). In the initial stages of proliferation, all EGL cells seem to partake in the synthesis of DNA and cell division. In the nascent cerebellum, the EGL has a great restitutive power.

In this experiment, the proliferation of the EGL following maternal Trp administration could be expressed through a rise in the speed of cell division and multiplication in the quantity of germinal cells. One of the features of the cells of the EGL is their overall capacity to proliferate and persist after birth. This present study agrees with Woolley III *et al*., 1974 who stated that Math1+ cells expressed Ki-67 (mitotic marker) at elevated rates: 50–60% on E13.5, >90% on E16.5 and >80% on day 9 and even after day 11 in the EGL anlage.

The mechanism by which tryptophan exerts its influence on the proliferation of the GCs and DNA synthesis presence of protein synthesis remains unclear. Alongside its role as a precursor of protein, tryptophan is a metabolic precursor of NAD and it could be deemed that some of the outcomes observed are mediated via unavailability of NAD (Woolley *et al*., 1974).

Nerve cells of the EGL possess terrific capacities to increase greatly, enough to replace GCs in the cerebellum after a premature X-irradiation exposure (Altman *et al.*, 1967). The ability of a cell to recover after an injury is available in the germinal compartment of the cerebellum. This reparative process might be responsible for developing a cerebellum with typical structure, form and size in the animals exposed to radiation (Altman *et al.*, 1968). A cerebellar developmental regeneration is possible (Altman *et al.*, 1969).

Substances which are capable of improving growth of cells within the CNS include IGF-I which is a large peptide that enhances maturation of the CNS (Folli *et al.*, 1996). Along with its receptor, a temporary expression is shown in some portions of the brain aligning with the periods of excessive growth of the axon, dendritic growth, and formation of synapses (Bondy, 1991). IGF-I kindles the multiplication of parent cells, stimulates the conversion of oligodendrocytes into mature cells, and improves the continued existence of these cells and oligodendrocytes *in vitro* (Werther *et al.,* 1993).

In genetically altered mice that overproduce IGF-I in the CNS, there was a noticeable gain in the magnitude of the brain (Mathews *et al.*, 1988), this is as a result of a visible increment in the quantity of nerve cells (Behringer *et al*., 1990). It is observed that IGF-I allows hippocampal dentate gyrus to grow and expand by boosting regeneration of nerve cells and synapses after delivery.

In the genetically altered IGF-II/IGF-I mice, a rise in the overall quantity of nerve cells in the IGL was noticed, commencing at PND 14 which continues into juveniles at PND 130. This

follows an earlier study that showed an amplified quantity of cells in the brainstem and cerebellum of the same mice (Dentremont *et al.*, 1999).

Studies have been conducted on conditions that can trim down the rate at which cells undergo division in the EGL. The rate at which GCs proliferated was quantified in the cerebellum of Down syndrome mouse model at PND 0, 3 and 7 at a period when GCPs divide in the EGL using an antibody against Ki67 (Kee *et al.*, 2002). The ratio between cells undergoing mitosis to the entire cells was taken as the few cells which stained positive for Ki67. They observed a decline in the ratio in the EGL of Down syndrome mice models at PND 0. Their finding is comparable to the 22% decline observed in the cerebellum of another model of Down syndrome mice at birth (Roper *et al.*, 2006). Put together these data proposes a parallel early activity that takes place at the time of delivery that influences GC division in the mouse replicas of cerebellum in Down syndrome. In another related study, a 32.6% reduction of GCP proliferation only at delivery was reported. At this period GCs multiply, travel inwardly to the interior of the cerebellum, and change into future cells (Laffaire *et al.*, 2009).

5.11 BCL-2 Apoptotic Cells

Many neurons that were initially generated die by a typical cell death that is predetermined as the the cells develop in the CNS in vertebrates. This is a frequent occurrence in the CNS and essential for typical neural developmental processes while the molecular mechanism responsible for this process remains unclear. In the wild type mice, the BC / SC which are produced in the medullary body in the first fifteen days migrate to the ML, their number is controlled by apoptosis (Yamanaka *et al.*, 2004). Apoptosis is an example of cell death that was programmed which has been speculated to take place after deficiency of NGF in sympathetic neurons (Edwards *et al*., 1991). It was reported that cerebellar granule neurons which are fully differentiated pass through the process of apoptosis when the concentrations of K^+ is reduced extracellularly. Morphological changes which are typical of death by apoptosis and breaking up of DNA, a vital feature, is seen in dead nerve cells which precedes the death process. Expression of specific genes is neccessary for suppressing the transcription and translation factors which inhibit apoptosis in cerebellar GCs induced by small quantities of K^+.

In mammals, the nervous system relies so much on nourishment for adequate existence (Barde, 1989). Among these substances known to have a positive effect on maturation of nerve cells and the ability to continue to live are the neurotrophins (Knusel *et al*., 1990).

Factors that can prevent death of GCs by reduced K^+ include IGF-I and cAMP. Others are BDNF and NT-3 which boosted the continued existence of young cerebellar GCs located in rat embryonic cerebella. BDNF is very dynamic on the young GCs in the EGL while NT-3 affects full-grown GCs (Segal *et al.*, 1992).

In this study, Trp was able to prevent apoptosis in the GCs in vivo. It may be speculated that the mechanisms underlying apoptosis caused by reduced K^+-mediated process of GCs are comparable to the type seen when neurons die in vivo in development and in vitro by deprivation of some factors. Because of their large number, GCs present an outstanding major system of cell culture to study the processes responsible for apoptosis in the CNS. Cerebellar GCs are a suitable structure to compare the processes responsible for the disintegration that occur in the CNS.

Bcl-2 is an important factor in mammals which shield against apoptosis and linked to K^+. The bcl-2 gene is generally produced as CNS develops (Merry *et al*., 1994). Acting as a remedy to apoptosis, Bcl-2 rescues cells that are fated to die by apoptosis and is useful in the profile of neural structures and in establishing circuits around neurons (Korsmeyer, 1993).

The result of this study demonstrated that group III and IV were protected from apoptosis by Bcl-2 expression as Trp was able to offer some neuroprotection against apoptosis. This result agrees with Zanjani *et al.,* 1996 who stated that overexpression of Bcl-2 may protect some Purkinje cells and prevent naturally occurring death of cells in olivary neurons (Zanjani *et al*., 1998).

Cerebellar GCs are part of the numerous cells that are recognized to rely on elevated K^+ to prevent them from dying when the cells are experimented on outside the body (Gallo *et al.*, 1990), and these cells go through cell death when the level of K^+ is reduced. Death of these cells is accompanied by structural modifications that indicated that dying was by programmed cell death. Disintegration of DNA, a characteristic of apoptosis, is seen in cells that are dying.

Of note, apoptosis of grown GCs is the outcome of these cells not been able to make appropriate contacts at the synapses in the newly formed ML, and, in the controls, programmed cell death

have an effect on GCs that have transited to the IGL when communications amid the parallel fibers and the PCs been set up (Lossi *et al.,* 2002).

Cerebellar granule cells rely on IGF-I for their existence. Nonetheless, the methods beneath these neuroprotection are not clear at the moment (Linseman *et al.,* 2002). Also, IGF-I is acknowledged to rescue the main cells from dying when stimulated by removal of factors of nourishment (Russell *et al.*, 1998); when cells are damaged by the overactivations of receptors for glutamate (Tagami *et al.*, 1997), and when there is an imbalance between free radicals and antioxidant (Heck *et al*., 1999). IGF-I is needful for the continued existence of CNS cells *in vivo* and *in vitro* (Ye *et al.,* 1996). Cerebellar granule cells are really reliant on IGF-I to prevent them from dying (Lin and Bulleit, 1997). As reported by Ye *et al*., 1996, primary IGF-I shields GCs from death of cells *in vivo*.

Also, death of GCs stimulated by reduced K^+ can be averted by IGF-I which produces active binding proteins in cerebellar GCs (Calissano *et al.*, 1993), proposing an activity for IGF-I in the maturation of these nerve cells. Cerebellar GCs do not turn out IGF-I at any stage, rather it is produced by PCs which produce this protein immediately after birth (Bondy, 1991). Between PND 4-10, young GCs move through the PCL to reach the IGL, wherein they differentiate. It might be that the IGF-I synthesized by the PCs is absorbed by the GCs and regulates the final maturation of these cells. Without a doubt, GCs migrating through the ML briefly exhibit immunoreactivity to IGF-I (Andersson *et al.*, 1988).

CHAPTER SIX

SUMMARY AND CONCLUSIONS

6.1 Summary

In this research, the difference between brain Trp level of pups in group II and III was considerably greater than group I. The difference in the serum Trp of the pups in group II was also greater and significant than group I. The difference occurred with no and/or low Trp administration and high dosages of Trp. Higher doses of Trp administration was quickly metabolized leaving traces in the brain and serum.

Trp in the serum enters the brain through the BBB by competing with LNAA. Low administration of Trp led to high serum and brain Trp levels; 100mg might be assumed the optimal dose for Trp administration especially for the persistence of the granule cells.

No physical abnormalities were observed in pups fed high doses of Trp, however, the animals recorded low brain and cerebellar weight consistently probably due to Trp toxicity.

At low doses of Trp granule cells persist in the EGL while at a high dose Trp is found to increase cell multiplication and movement of the GCs into the IGL.

This study showed there was no change in the cerebellar cortical layer thickness of all the animals. As already documented, the peak EGL thickness occurred on day 7 in all groups with no difference between the groups in addition to Trp administration. However, Trp did have a positive effect on the EGL in the second week with respect to a larger EGL. Also, the mean density of the granule cells appeared the same.

The molecular and internal granular layer lengths increased from postnatal day PND 0 in all the groups. The EGL initially grew rapidly, climaxed on postnatal day 7 and slowed down its growth due to cessation of mitosis between the dividing cells.

More mitotic cells and pyknotic cells were seen in 300mg dosage. This could probably be due to an assumption that Trp enhances cell division and may thus be used as a mitogenic agent. There is a probability that the more cells are produced the more cells will be dying and this might account for more granule cell death observed amidst the migrating cells in the ML. More radially oriented granule cells following Trp administration indicating migration were observed migrating in the nascent ML of all the experimental groups while less migrating cells were seen in control group.

Bergmann glial cells of control animals showed excess cytoplasmic excrescenses as the processes are all pial-directed, which were abundant in contrast to the Trp groups. The disappearance of the processes of Bergmann glia cells which were pial directed were slowed down (delayed maturation) with low doses of Trp.

High percentage positivity of ki-67 proliferating cells was seen in the Trp administered group indicating that the EGL could be a potential site for neurogenesis. Using Bcl-2 marker, Trp seemed to have a neuroprotective role against apoptotic cells as evidenced by reduced dead cells found within the EGL.

6.2 Difference from Previous Researches

Unlike other Trp studies which centered on metabolism of Trp and rate limiting enzymes, my experiments included the following:

- The use of analytical grade of Trp instead of a Trp- deficient diet which still delivered the necessary effects on the animals.
- The EGL thickness and density were measured as opposed to other studies
- Activities done on the EGL, Trp and GC movement is rare.
- The use of proliferating cell nuclear assay (PCNA) especially Ki-67 in relation to EGL and Trp has been rare too.

6.3 Contributions to Knowledge

The EGL is a vital structure for studying how neurons develop and its importance as a probable source brain cancers in children cannot be over emphasized (Kool *et al.*, 2008). The EGL has been found to be a potential neurogenic site which could lead to increased neural plasticity and benefit stem cell research. Trp has been shown to induce cell division and can thus be used as a mitogenic agent in the EGL.

- Trp deficiency leads to a delayed persistence of the EGL.
- Availability of Trp leads to increased multiplication and movement of GCs and a quick transformation into IGL and we may thus report that the growth pattern of the CC is associated with the migration of neurons derived in great numbers from the subpial EGL.
- With the use of in vivo and in vitro generations of GCs from embryonic cells with the ability to develop into specialized types, these cells will be useful in the management and

progresses made in the evolvement of drug therapy for diseases of the cerebellum such as spinocerebellar degeneration and medulloblastoma which originate in the EGL and besides the early differentiation of cerebellar nerve cells.

- Using cerebellar cells that are engineered genetically, embryonic stem cells will be a prospective means for a fast and vast investigation not forgetting the extensive motility of the nerves in the EGL.
- Granule cells have been often used to study toxicity in the CNS, which is assumed to take place through necrosis. The mode by which these cells die discretely involves distinct processes. Cerebellar granule cells are suitably used to study the comparison and disparity in the molecular mechanisms responsible for these degenerative conditions.
- Bearing in mind the large quantity of cerebellar GCs, this may offer an exceptional system of culturing cells primarily in order to explore the processes fundamental to death of nerve cells in the CNS.

6.4 Recommendations

It is recommended that:

- Trp should not be deficient in the diet or may be supplemented in moderate doses for the infant
- The EGL is a known site for neurogenesis and a possible area of stem cell research
- Trp can be employed as a mitogenic agent
- Genes that regulate migration in the EGL should be studied in relation to Trp.

REFERENCES

Abbott, L. C., Jacobowitz, D. M. 1995. Development of calretinin immunoreactive unipolar brush-like cells and an afferent pathway to the embryonic and early postnatal mouse cerebellum. *Anat. Embryol. (Berl).* 191 (6) 541-559

Adams, N. C., Tomoda, T., Cooper, M., Dietz, G. and Hatten, M.E. 2002. Mice that lack astrotactin have slowed down neuronal migration. *Development,* 129 (4): 965-72

Albay III, R., Chen, A., Anderson, G.M., Tatevosyan, M and Janušonis, S. 2009. Relationships among body mass, brain size, gut length, and blood tryptophan and serotonin in young wild-type mice*BMC Physiology*, 9:4 doi:10.1186/1472-6793-9-4

Alcantara, S., Ruiz, M., De Castro, F., Soriano, E., Sotelo, C. 2000. Netrin 1 acts as an attractive or as a repulsive cue for distinct migrating neurons during the development of the cerebellar system. *Development* 127 (7):1359 –1372.

Alder. J., Cho, N., Hatten, M. E. 1996. Embryonic precursor cells from the rhombic lip are specified to a cerebellar neuronal identity. *Neuron* 17:389 –399.

Alegría, A., Barberá, R., Farré, R, Ferrerés, M., Lagarda. M. J. 1996. Isocratic high-performance liquid chromatographic determination of tryptophan in infant formulas, *J. Chromatogr. A, 72:*83–88.

Altman, J., and Das, G. D. 1967. Postnatal Neurogenesis in the Guinea Pig. *Nature* (London) 214:1098-1101

Altman, J., Anderson, W.J. and Wright, K. A. 1968. Gross morphological consequences of irradiation of the cerebellum in infant rats with repeated doses of low-level x-ray. *Exp. Neurol.*, 21 (1): 69-91.

Altman, J. 1969. Handbook of Neurochemistry. Vol. II: *Structural Neurochemistry*137-182. Plenum Press,New York & London

Altman, J., Anderson W.J., and Wright, K. A. 1969. Reconstitution of the external granular layer of the cerebellar cortex in infant rats after low-level X-irradiation. *Anat.Rec.*, 163 (3): 453-471

Altman, J. 1972. Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J. Comp.Neurol*.145 (3) 353-397

Altman, J. and Bayer, S. A. 1978. Prenatal development of the cerebellar system in the rat. I. Cytogenesis and histogenesis of the deep nuclei and the cortex of the cerebellum. *Journal of Comparative Neurology* 179 (1) 23-48.

Altman, J. 1982. Morphological development of the rat cerebellum and some of its mechanisms. *Exp Brain Res* 6:8–46.

Altman, J., and Bayer, S. A. 1985a. Embryonic development of the rat cerebellum. I. Delineation of the cerebellar primordium and early cell movements. *J. Comp. Neurol.* 231 (1) 1–26. doi: 10.1002/cne.902310103

Altman, J. and Bayer, S. A. 1997. Development of the Cerebellar System: In Relation to its Evolution Structure and Functions. 1st Edition ISBN-13: 978-0849394904 New York: CRC Press.

Altria, K. D., Harkin P., Hindson M. G. 1996. Quantitative determination of tryptophan enantiomers by capillary electrophoresis, *J. Chromatogr. Biomed.* 686 (1):103–110.

Andersson, I. K., Edwall, D., Norstedt, G., Rozell, B., Skottner, A. and Hansson, H. A.. 1988. *Acta. Physiol. Scand.* 132, 167-173.

Arevalo R, Afonso D, Castro R., Rodriguez M. 1991. Fetal brainbserotonin synthesis and catabolism is under control of mother intake of Tryptophan. *Life Sci* 49: 53 – 66

Atkins, J.F. and Gesteland, R. 2002. The 22nd Amino Aci. Science vol. 296(5572):1409-10

Attwell, P. J., Cooke, S. F. and Yeo C. H. 2002. Cerebellar function in consolidation of a motor memory; *Neuron*34: 1011–1020

Badawy, A. A- B. 1988. Effects of pregnancy on tryptophan metabolism and disposition in the rat. *Biochemical Journal* 255: 369-372

Badawy, A. A- B. 2002. Tryptophan metabolism in alcoholism. *Nutr. Res. Rev.* 1:123–152.

Badawy, A. A-B. 2015. Tryptophan metabolism, disposition and utilization in pregnancy. Biosci. Rep. 35(5):e00261

Bähr, M., 2000. Live or let die—retinal ganglion cell death and survival during development and in lesioned CNS. *Trends Neurosci.* 23, 483– 490.

Bailey, K., Rahimi Balaei, M., Mannan, A., Del Bigio, M. R., and Marzban, H. 2014. Purkinje cell compartmentation in the cerebellum of the lysosomal Acid phosphatase 2 mutant mouse (nax—naked-ataxia mutant mouse). PLoSOne 9:e94327.

Barde, Y. A. 1989.Trophic factors and neuronal survival. *Neuron* 2, (6): 1525-34. Doi:10.1016/0896-6273(89)90040-8. PMID:2697237

Batistatou, A., Merry, D. E., Korsmeyer, S. J., Greene, L.A. 1993. Bcl-2 affects survival but not neuronal differentiation of PC12 cells. *J. Neurosci* 13:4422– 4428.

Bazwinsky, I., Härtig, W., Rübsamen, R. 2008. Characterization of cochlear nucleus principal cells of Meriones unguiculatus and Monodelphis domestica by use of calcium-binding protein immunolabeling. *J Chem Neuroanat.* 35:158–74.

Beas-Za´rate, C., Del Angel-Meza, A.R., Morales-Villagra´n, A., FeriaVelasco, A., 1988. Serotonin uptake in the central nervous system of rats fed a corn diet. *Comp. Biochem. Physiol.* 89C, 173–177.

Behringer, R.R., Lewin, T.M., Quaife, C.J., Palmiter, R.D., Brinster, R.L., D'Ercole, A. J. 1990. Expression of insulin-like growth factor I stimulates normal somatic growth in growth hormone-deficient transgenic mice. *Endocrinology* 127: 1033-40

Bell C, Abrams J, Nutt D. 2001. Tryptophan depletion and its implications for psychiatry. *Br J Psychiatry*; 178:399–405.

Bell, J. E., Sandison, A., Boddy, J., Franks, A. J., Batcup, G., Calvert, R., Gordon, A. 1989. Development of the cerebellum with particular reference to cellular differentiation in the external granular layer. *EarlyHum.Dev.* 19 (3): 199–211.

Bellamy, T. C. 2006. Interactions between Purkinje neurons and Bergmann glia. *Cerebellum* 5, 116–126.doi:10.1080/14734220600724569

Bender, D. A. 1983. Biochemistry of tryptophan in health and disease. *Mol. Aspects*. Med.6:1–97.

Bernier, P. J. and Parent, A. 1991. Bcl-2 Protein as a Marker of Neuronal Immaturity in Postnatal Primate Brain *J. Neurosci.18*(7):2486–2497

Berthié, B., Axelrad, H. 1994. Granular layer collaterals of the unipolar brush cell axon display rosette-like excrescences. A Golgi study in the rat cerebellar cortex. *Neurosci Lett.* 167:161–5.

Bishop, G.A., Ho, R.H., King, J.S., 1985. An immunohistochemical study of serotonin development in the opossum cerebellum. Anat. Embryol. 171, 325–338.

Blair, L.A.C., Bence Hanulec, K.K., Mehta, S., Franke, T., Kaplan, D., Marshall, J. 1999. Akt-dependent potentiation of L channels by insulin-like growth factor –I is required for neuronal survival. J. Neurosci 19:1940-51

Blaschke, A. J., Weiner, J. A., Chun, J. 1998. Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system. *J Comp Neurol.* 396:39–50.

Bloxan, D. L., Curzon, G., Kantamanemi, B. D. and Tricklebank, M. D. 1977. Effects of Tryptophan and Portocaval Anastomosis on Activity and Brain Tryptophan Metabolism. Proc. of the BPS, 30th, 31 March 277P.

Bondy, C. 1991. Transient IGF-I gene expression during maturation of functionally related central projection neurons *J. Neurosci* 11:3442-55.

Booth, D. G., Takagi, M., Sanchez-Pulido, L., Petfalski, E., Vargiu, G., Samejima, K., Imamoto, N., Ponting, C.P., Tollervey, D, Earnshaw, W. C. and Vagnarelli, P. 2014. Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. *eLife* 3:e01641.

Borges-Merjane C., Trussell L.O. 2015. On and off unipolar brush cells transform multisensory inputs to the auditory system. *Neuron,* 85:1029-42

Bouslama-Oueghlani, L., Wehrlé, R., Doulazmi, M., Chen, X. R., Jaudon, F., Lemaigre-Dubreuil, Y., Rivals, I., Sotelo, C., DuSart, I. 2012. Purkinje cell maturation participates

in the control of oligodendrocyte differentiation: role of sonic hedgehog and vitronectin. PLoS One. 7(11):e49015.

Brezun J., M., Daszuta A. 2000. A depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. *Neuroscience.* 89:999-1002.

Brilliger, D. 1984. The collected works of John W. Tukey: More Mathematical 1938-1984 (Wadsworth & Brooks/ Cole Statistics/ Probability Series) ISBN-13: 978-0534051037, ISBN-10:0534051030

Bucci, L. and Chiavarelli, R. 1979. The Toxic Effects of Long Term Oral Administration of L-Tryptophan in Rats with Portocaval Shunt. *Br. J. exp. Path.,* 60 (4): 389-394.

Budavari, S., O'Neil M. J., Smith, A., Heckleman, P. E., Kinneary, J. F. 1996. The Merck Index. An encyclopedia of chemicals, drugs and biological. 12th Ed, Merck & Co. Whitehouse Station, NJ,USA ISBN 09119910-12-3. V, NI-360 pp.

Bush, E. C., and Allman, J. M. 2003. The scaling of white matter to gray matter in cerebellum and neocortex. *Brain Behav. Evol* .61, 1-5

Calissano, P., Ciotti, M.T., battistini, L., Zona, C., Angelini, A., Merlo, D. and Mercanti, D. 1993. Proc. *Natl. Acad. Sci.* USA 90, 8752- 56

Caston, J., Hilber, P., Chianale, C. and Mariani, J. 2003. Effect of training on motor abilities of heterozygous staggerer mutant (Rora (+) / Rora (sg) mice during aging; Behav. *Brain Res.*141(1):35-42

Castre´n, E., Ohga, Y., Berzaghi, M. P., Tzimagiorgis, G., Thonen, H., Lindholm, D. 1994. Bcl-2 messenger RNA is localized in neurons of the developing and adult rat brain. *Neuroscience* 61:165–177.

Cavanagh, J. B. and Htu Kyu, M. A. 1971. Type II Alzheimer Change Experimentally Produced in Astrocytes in the Rats. *J. Neurol. Sci.,* 12(1): 63-75.

Cepko, C.L., Golden, J.A., Szele, F.G., Lin, J.C. 1997. Lineage analysis in the vertebrate central nervous system. In: Molecular and cellular approaches to neural development (Cowan WM, Jessel T, Zipursky SL, eds). London: Oxford UP. Cerebellar Cortex-Cytology and Organization

Chanas-Sacre, G., Rogister, B., Moonen, G., Leprince, P. 2000. Radial glia phenotype: Origin, regulation and transdifferentiation. *J. Neurosci. Res.* 61:357-363

Chédotal, A. 2010. Should I stay or should I go? Becoming a granule cell. *Trends Neurosci.*33, 163–172.

Chen, Y., Guillemin, G. J. 2009. Kynurenine pathway metabolites in humans: disease and healthy States. *Int. J Tryptophan Res.* 2:1–19. [PubMed: 22084578]

Cheutin, T., O'Donohue, M. F., Beorchia, A., Klein, C., Kaplan, H., Ploton, D. 2003. Three-dimensional Organization of pKi-67: A Comparative Fluorescence and Electron

Tomography Study Using Fluoronanogold. *Journal of Histochemistry & Cytochemistry* 51:1411–1423.

Choi, B. H., and Lapham, L. W. 1980. Evolution of Bergmann glia in developing human fetal cerebellum: a Golgi, electron microscopic and immunofluorescent study. *Brain. Res.* 190, 369–383.doi:10.1016/0006-8993(80)90280-2

Christensen, H. N. 1979. Exploiting amino acid structure to learn about membrane transport. *Advances in Enzymology* **49**, 41–101.

Chuong, C. M. 1990. Differential roles of multiple adhesion molecules in cell migration: granule cell migration in cerebellum. *Experientia* 46:892– 899.

Chuong, C., M., Crossin, K. L., Edelman, G. M. 1987. Sequential expression and differential function of multiple adhesion molecules during the formation of cerebellar cortical layers. *J Cell Biol* 104:331–342.

Cioni, P., Strambini, G.B. 2002. Tryptophan phosphorescence and pressure effects on protein structure, *Biochim. Biophys. Acta,1595:*116–130.

Clos, J., Favre, M., Selme-Matrat, M., Legrand, J. 1977. Effects of undernutrition on cell formation in the rat brain and specially on cellular composition of the cerebellum. *Brain research* 123(1): 13-26

Clos, J., Legrand C., Legrand J. 1980. Effects of thyroid state on the formation and early morphological development of Bergmann glia in the developing rat cerebellum. *Dev., Neurosci.* 3:199-208

Comai, S., Bertazzo, A., Carretti, N., Podfigurna-Stopa, A., Luisi S and Costa C. V. L. 2010. Serum Levels of Tryptophan, 5-Hydroxytryptophan and Serotonin in Patients Affected with Different Forms of Amenorrhea. *International Journal of Tryptophan Research* 2010:3 69–75

Coramira, N. O., Seve, B., Lebreton, Y., Ganier, P. 1991. Effect of dietary tryptophan on muscle, liver and whole body protein synthesis in weaned piglets relationship to plasma-insulin. *British Journal of Nutrition* 66:423-435

Corrales, J. D., Rocco, G. L., Blaess, S., Guo, Q., Joyner, A. L. 2004. Spatial pattern of sonic hedgehog signaling through Gli genes during cerebellum development. *Development,*131:5581-5590.

Corrales, J. D., Blaess, S., Mahoney, E. M., Joyner, A. L. 2006. The level of sonic hedgehog signaling regulates the complexity of cerebellar foliation. *Development* 133:1811–1821.

Craigie, E. H. 1938. The comparative anatomy and embryology of the capillary bed of the central neuron system. *Proceedings of the Association for Research into Nervous and Mental diseases.* 18: 3-28.

Cubero, J., Valero, V., Sánchez, J., Rivero, M., Parvez, H., Rodríguez, A.B and Barriga, C. 2005. The circadian rhythm of tryptophan in breast milk affects the rhythms of 6-

sulfatoxymelatonin and sleep in newborn Neuroendocrinology Letters No.6 December Vol. 26 (6): 657-61 PMID: 16380706

Cunningham, T. J., Mohler, I. M. and Giordano, D. L. 1982. Naturally occurring neuron death in the ganglion cell layer of the neonatal rat: Morphology and evidence for regional correspondence with neuron death in the superior colliculus. *Dev. Brain Res.* 2: 203-215.

D'Angelo, E. and Casali, S. 2013. Seeking a unified framework for cerebellar function and dysfunction: from circuit operations to cognition *Frontiers in Neural Circuits.* 6 (116):1-23

Davies, A. M. 1995. The Bcl-2 family of proteins, and the regulation of neuronal survival. *Trends Neurosci.* 18:355–358.

De Marte M. L. and Enesco H. E. 1985. Influence of diet on plasma tryptophan and brain serotonin levels in mice. *Experiential*, 41, 1:48-50

De Zeeuw, C. I., and Berrebi, A. S. 1995. Postsynaptic targets of Purkinje cell terminals in the cerebellar and vestibular nuclei of the rat. *Eur. J. Neurosci.* 7, 2322–2333.

Del Angel-Meza, A. R., Tapia-Arizmendi, G., Feria-Velasco, A., 1984. Effects of food restriction during lactation on postnatal development of rat cerebellum. Correlative biochemical and structural study. *Nutr. Rep. Int.* 30, 95–109.

Del Angel-Meza, A. R., Beas-Za´rate, C., Morales-Villagra´n, A., 1989. Effects of corn-fed and protein restriction on rat cerebellum and brain stem maturation. *Nutr. Rep. Int.* 40, 1199–1206.

Del Angel-Meza A. R., Ramirez Cortes L, Olver-Cortes E, Perez Vega M. I, Gonzales- Burgos, I. 2001. A tryptophan-deficient diet corn-based diet induces plastic responses in cerebellar cortex cells of rat offspring distinct morphological structures to interact with cerebellar neurons. *Int. J. Devl Neuroscience*. 19: 447–453.

DelCerro, M., and Swarz, J. R. 1976. Prenatal development of Bergmann glial fibres in rodent cerebellum. *J. Neurocytol.* 5, 669–676.

Dentremont, K. D., Ye, P., D'Ercole, A. J. 1999. Increased insulin-like growth factor-I (IGF-I) expression during early postnatal development differentially increases neuron number and growth in medullary nuclei of the mouse. *Dev. Brain. Res* 114:135- 41

Diño, M. R., Schuerger, R. J., Liu, Y., Slater, N. T., Mugnaini, E. 2000. Unipolar brush cell: a potential feedforward excitatory interneuron of the cerebellum. *Neuroscience*.98:625–36.

Dobbing, J., Sands, J. 1973. Quantitative growth and development of human brain. *Arch Dis Child* 48:734–67

Dong X and Zou X. 2017. Effects of excess dietary tryptophan on laying performance, antioxidant capacity and immune function of laying hens *IntechOpen*. 249-257

Dong-Ryul, L., Kondo, H., Furukawa, S., Nakano, K. 1997. Stimulation of NGF production by tryptophan and its metabolites in cultured mouse astroglial cells. *Brain Res*. 777: 228–30.

Doyle, D. 1967. Portocaval Anastomosis in Rats. M.D. Thesis, University of Edinburgh, Edinburgh.

Edmondson, J.C., Liem, R. K., Kuster, J. E., Hatten, M. E.1988. Astrotactin, a novel cell surface antigen that mediates neuron-glia interactions in cerebellar microcultures. *J Cell Biol* 106:505–517.

Edwards, S. N., Buckmaster, A. E. and Tolkovsky, A. M. 1991. The death programme in cultured sympathetic neurons can be suppressed at the posttranslational level by the nerve growth factor cyclic AMP and depolarization. *J. Neurochem.* 57, 2140-2143.

Endl, E., Gerdes, J. 2000b. The Ki-67 Protein: Fascinating Forms and an Unknown Function. *Experimental Cell Research* 257:231–237.

Englund, C., Kowalczyk, T., Daza, R. A., Dagan, A., Lau, C., Rose, M. F., Hevner, R. F. 2006. Unipolar brush cells of the cerebellum are produced in the rhombic lip and migrate through developing white matter. *J. Neurosci.* 26 (36): 9184-9195. doi: 10. 1523/jneurosci.1610-06.2006

Fahrion, J.K.; Komuro, Y.; Ohno, N.; Littner, Y.; Nelson, C.; Kumada, T.; Komuro, H. 2013. Cerebellar Patterning. In Comprehensive Developmental Neuroscience: Patterning and Cell Type Specification in the Developing CNS and PNS, 1st ed.; Rubenstein, J., Rakic, P., Eds.; Elsevier Inc.: Oxford, UK; Volume 1, pp. 211–225.

Fernstrom, J. D., Fernstrom, M. H. 2006. Exercise, serum free tryptophan, and central fatigue. *J Nutr*. 136(2):553S–559S.

Fernstrom, J.D. 2013. Large neutral amino acids; dietary effects on brain neurochemistry and function. *Amino Acids* 45 (3):419-430

Feurte, S., Gerozissis, K., Regnault, A., and Paul, F. M. 2001. Plasma Trp/LNAA ratio increases during chronic ingestion of an alpha-lactalbumin diet in rats. *Nutr. Neurosci*., 4(5): 413-8.

Fisher, R. 1992. Statistical Methods for Research Workers. Springer-Verlag, New York ISSN 1492-3173

Fleming, J. T., He, W., Hao, C., Ketova, T., Pan, F.C., Wright, C. V., Litingtung, Y., Chiang, C. 2013. The Purkinje neuron acts as a central regulator of spatially and functionally distinct cerebellar precursors. *Dev. Cell.* 27: 278–92.

Fligny, C., Fromes, Y., Bonnin, P., Darmon, M., Bayard, E., Launay, J. M., Cote, F., Mallet, J., Vodjdani, G. 2008. Maternal serotonin influences cardiac function in adult offspring. *FASEB J.* 22:2340-2349.

Folli, F., Ghidella, S., Bonfanti, L., Kahn, C.R., Merighi, A. 1996. The early intracellular signaling pathway for the insulin/insulin-like growth factor receptor family in the mammalian central nervous system. *Mol. Neurobiol.* 13:155-83

Fox, C. A. 1962. The structure of the cerebellar cortex. In E. C. Crosby, T. Humphrey and E. W. Laurer (ed.), Correlative anatomy of the nervous system. MacMillan, New York, p. 193-198.

Fujita, S. 1964. Analysis of neuron differentiaton in the central nervous system by tritiated thymidine autoradiography. *J.Comp Neurol.* 122 (3):311-327

Fujita, S., Shimada, M. and Nakamura, T. 1966. Tritiated thymidine autoradiographic studies of the cell proliferation and differentiation in the external and internal granular layers of the mouse cerebellum. Journal of comparative *Neurology* 128: 191-208

Fujita, S. 1967. Quantitative analysis of cell proliferation and differentiation in the cortex of the postnatal mouse cerebellum. *The Journal of Cell Biology*. Vol. 32. pg 277-287

Funakoshi, H., Kanai, M., and Nakamura, T. 2011. Modulation of Tryptophan Metabolism, Promotion of Neurogenesis and Alteration of Anxiety-Related Behavior in Tryptophan 2, 3-Dioxygenase-Deficient Mice. *International Journal of Tryptophan Research*: 4: 7–18

Galas, L., Bénard, M., Lebon, A., Komuro, Y., Schapman, D., Vaudry, H., Vaudry, D., and Komuro, H. 2017. Postnatal Migration of Cerebellar Interneurons. Review. *Brain Sciences* 7, 62: 1-18

Gallo, V., Giovanini, C. and Levi, G. 1990. Modulation of non-N-methyl –D-aspartate receptors in cultured cerebellar granule cells. *J. Neurochem.* 54(5) 1619-1625.

Ganapathy, M. E., Leibach, F. H., Mahesh, V. B., Howard, J. C., Devoe, L. D. and Ganapathy, V. 1986. Characterization of tryptophan transport in human placental brush-border membrane vesicles. *Biochemical Journal* 238, 201–208.

Gaspar, P., Cases, O., Maroteaux, L. 2003. The developmental role of serotonin: news from mouse molecular genetics. *Nat. Rev. Neurosci*. 4:1002-1012.

Gerdes, J., Schwab, U., Lemke, H., Stein, H. 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *InternationalJournal of Cancer* 31:13–20.

Gerdes, J., Li, L., Schlueter, C., Duchrow, M., Wohlenberg, C., Gerlach, C., Stahmer, I. Kloth, S Brandt, E., Flad, H. D. 1991. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 138(4):867-73

Gershoff, S. N., Gill, T. J., Simonian, S. J., Steinberg, A. I. 1968. Some effects of amino acids deficiencies on antibody formation in the rat. *Journal of Nutrition* 95:184-189

Gillilan, L. A. 1969. The arterial and venous blood supplies to the cerebellum of primates. *Journal of Neuropathology and Experimental Neurology*. 28, 295-297

Goodrich, L. V., Milenkovic, L., Higgins, K. M., Scott, M. P. 1997. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* 277:1109 –1113.

Griffin, W. S, Woodward, D. J and Canda R. 1977. Malnutrition and brain development: cerebellar weight, DNA, RNA, protein and histological correlations. *Journal of Neurochemistry* 28(6)1269-1279

Grimaldi, P., Parras, C., Guillemot, F., Rossi, F., Wassef, M. 2009. Origins and control of the differentiation of inhibitory interneurons and glia in the cerebellum. *Dev Biol.* 328:422–33.

Grosche, J., Matyash, V., Moller, T., Verkhratsky, A., Reichenbach, A., Kettenmann, H. 1999. Microdomains for neuron-glia interaction: Parallel fiber signaling to Bergmann glial cells. *Nat. Neurosci.* 2:139–143.

Grosche, J., Kettenmann, H., Reichenbach, A. 2002. Bergmann glia form distinct morphological structures to interact with cerebellar neurons. *J. Neurosci. Res.* 68:138–149.

Gutierrez, C. I, Urbina, M., Obregion, F., Glykys, J., Lima, L. 2003. Characterization of tryptophan high affinity transport system in pinealocytes of the rat. Day-night modulation. *Amino Acids*. 25:95–105.

Hadj-Sahraoui, N., Frederic, F., Zanjani, H., Delhaye-Bouchaud, N., Herrup, K. and Mariani, J. 2001. Progressive atrophy of cerebellarPC dendrites during aging of the heterozygous staggerer mouse (Rora+/sg).*Dev. Brain Res.*126: 201–209

Hallonet, M., and Alvarado-Mallart, R. M. 1997. The chick / quail chimeric system: a model for early cerebellar development. *Perspect. Dev. Neurobiol.* 5: 17–31.

Hanaway, J. 1967. Formation and differentiation of the external granular layer of the chick cerebellum. *J. Comp. Neurol.* 131:1–14.

Hartmann, W. 2005. Insulin-like growth factor II is involved in the proliferation control of medulloblastoma and its cerebellar precursor cells. *Am. J. Pathol.*166:1153–1162.

Hashimoto, M., and Hibi, M. 2012. Development and evolution of cerebellar neural circuits. *Dev. Growth Differ.*54(3): 373–389.

Hatten, M. E., Liem, R. K. H. and Mason, C. A. 1984. Defects in specific associations between astroglia and neurons occur in microcultures of weaver mouse cerebellar cells. *J. Neurosci.* 4:1163-1172

Hatten, M. E., and Mason, C. A., 1990. Mechanisms of glial-guided neuronal migration in vitro and in vivo. *Experientia* 46: 907–916.

Hatten, M. E., Heintz, N. 1995. Mechanisms of neural patterning and specification in the developing cerebellum. *Annu. Rev. Neurosci.* 18:385– 408.

Heck, S., Lezoualc'h, F., Engert, S., Behl, C. 1999. Insulin-like growth factors-1-mediated neuroprotection against oxidative stress is associated with activation of nuclear factor kB. *J. Biol Chem.* 274: 9828-9835

Heine, V. M., Maslam, S., Joe'ls, M., Lucassen, P. J. 2004. Prominent decline of new born cell proliferation, differentiation, and apoptosis in the aging dentate gyrus in the absence of an age-related hypothalamus-pituitary-adrenal axis activation. *Neurobiol. Aging* 25:361-75

Heinsen, H. and Heinsen, Y.L. 1983. Quantitative studies on regional differences in Purkinje cell dendritic spines and parallel fiber synaptic density. *Anat. Embryol. (Berl).* 168:361-70

Heng, X., Guo, Q., Leung, A. W., Li, J. Y. M. 2017. Analogous mechanism regulating formation of neocortical basal radial glia and cerebellar *Bergmann glia.* eLife 6:1-30

Herndon, R. M. 1964. The fine structure of the rat cerebellum. II . The stellate neurons, granule cells, and glia. The Journal of cell biology 23 (2), 277-293

Herndon R.M., Margolis, G and Kilham L. 1971. The synaptic organization of malformed cerebellum induced by perinatal infection with the felin pan leukopenia virus (PLV) II. The Purkinje cells and its afferents. *J. Neuropathol Exp. Neurol.* 30: 557 – 570

Herrero, E., Aragon, M. C., Gimenez, C., Valdivieso, F. 1983. Tryptophan transport into plasma membrane vesicles derived from rat brain synaptosomes. *J. Neurochem*. 40:332–337

Herrup, K., Kuemerle, B. 1997. The compartmentalization of the cerebellum. Annu Rev *Neurosci* 20:61–90.

Hery, R., Rouer, E., Kan, J.P. and Glowinski, J. 1974. The major role of the tryptophan active transport system in the diurnal variations of 5-hydroxytryptamine synthesis in the rat brain. *Adv. biochem. Psychopharmac.* 11:163-167.

Hibi, M., and Shimizu, T. 2012. Development of the cerebellum and cerebellar neural circuits. *Dev. Neurobiol.* 72, 282–301. doi:10.1002 /dneu. 20875

Hicks, S. P., and D'Amato C. J. 1961. How to design and build abnormal brains using radiation during development In. Disorders of the developing nervous system. Fields, W.S. Desmond, M. M. (eds), Repr.Nr. 244, Cancer Res. Inst. New England Deaconess Hosp.

Hillman, D. E., Chen, S., 1981. Vulnerability of cerebellar development in malnutrition. I. Quantitation of layer volume and neuron numbers. *Neuroscience* 6, 1249–1262.

Hockenbery, D. M., Zutter, M., Hickey, W., Nahm, M., Korsmeyer, S. J. 1991. Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci* USA 88:6961– 6965.

Hoerder-Suabedissen, A., Paulsen, O., Molnar, Z. 2008. Thalamocortical maturation in mice is influenced by body weight.*J Comp Neurol*511:415-420.

Hopewell, J.W. 1974.the permanent long term effects of X-irradiation on the rat cerebellum. *Acta neuropath.* (Berl.) 27: 163-169

Hsu, Y. H., Huang, H.Y., Tsaur, M.L. 2003. Contrasting expression of Kv4.3, an A-type K+ channel, in migrating Purkinje cells and other post-migratory cerebellar neurons. *Eur. J. Neurosci.* 18(3): 601-12.

Huang, X., Liu, J., Ketova, T., Fleming, J. T., Grover, V. K., Cooper, M. K., Litingtung, Y., Chiang, C. 2010. Transventricular delivery of Sonic hedgehog is essential to cerebellar ventricular zone development. *Proc Natl Acad Sci* USA 107(18):8422–8427.

Hyodo-Taguchi, Y., Fushiki, S., Kinoshita, C., Ishikawa, Y. and Hirobe, T. 1998. Effects of low dose X-irradiation on the development of the mouse cerebellar cortex. *J. Radiat. Res. (Tokyo)* 39 (1): 11-19.

Ilijic, E., Guidotti, A., Mugnaini, E. 2005. Moving up or moving down? Malpositioned cerebellar unipolar brush cells in reeler mouse. *Neuroscience.* 136 (3):633–47.

Indebir, S. 2014. Textbook of Human Neuroanatomy (Fundamental and Clinical) 8th Ed. Pg 147-160

Inouye M, Kameyama Y. 1983. Cell death in the developing rat cerebellum following X-irradiation of 3-100 rad: A quantitative study *J.Radiat Res.,* 24: 259-269

Iob, V., Matteson, W. J. JR, Sloan, N., Coon, W. W., Turcott, J. G. and Child, C. G. 1970. Alteraction in Plasma Free Amino Acids in Dogs with Hepatic Insufficiency. *Surg. Gyn and Obst.*, 130 (5): 794-800.

IUPAC-IUBMB Joint Commission on Biochemical Nomenclature. 2007. "Nomenclature and Symbolism for Amino Acids and Peptides". Recommendations on Organic & Biochemical Nomenclature, Symbols & Terminology etc. http://www.chem.qmul.ac.uk/iupac/Amino Acid

Jaarsma, D., van den Berg R, Wulf, P., van Erp, S., Keijzer, N., Schlager, M. A., De Graaff, E., De Zeeuw, C. I., Pasterkamp, R. J., Akhmanova, Hoogenrand, C.C. 2014. A role for Bicaudal-D2 in radial cerebellar granule cell migration. *Nature Communications* 5(1):1-13 doi: 10.1038/ncomms4411.

Jacobs, B., Johnson, N. L., Wahl, D., Schall, M., Mas eko, B. C., Lewandowski, A., Raghanti M.A, Wicinski, B., Butti, C., Hopkins, W.D., Bertelsen M.F., Walsh,T., Roberts, J. R., Reep, R. L., Hof, P. R., Sherwood, C.C and Manger P.R. 2014. Comparative neuronal morphology of the cerebellar cortex in afrotherians, carnivores, cetartiodactyls, and primates. *Frontiers in Neuroanatomy.* 8 (24):1-27

Jacobson, M. 1991. Time and sequence of origin of neurons and glial cells in the central nervous system. In: Jacobson M, ed. *Developmental neurobiology*. New York: Plenum Press, 58–64

Jankovski, A., Rosi, F., Sotelo, C. 1996. Neuronal precursors in the postnatal mouse cerebellum are fully committed cells: evidence from heterochronic transplantations. *Eur J Neurosci* 8:2308 –2319.

Janušonis, S., Anderson, G. M., Shifrovich, I., Rakic, P. 2006. Ontogeny of brain and blood serotonin levels in 5-HT1A receptor knockout mice: potential relevance to the neurobiology of autism. *J. Neurochem.*99:1019-1031.

Jensen, P., Smeyne, R., Goldowitz, D. 2004. Analysis of cerebellar development in math1 null embryos and chimeras. *J. Neurosci.* 24: 2202–11.

Jernigan, T. L., Archibald, S. L., Fennema-Notestine. C., Gamst, A. C., Stout, J. C., Bonner, J. and Hesselink, J. R. 2001. Effects of age on tissues and regions of the cerebrum and cerebellum. *Neurobiol. Aging*22:581–594

Kanai, M., Funakoshi, H., and Nakamura, T. 2010. Implication of Tryptophan 2, 3-Dioxygenase and its Novel Variants in the Hippocampus and Cerebellum During the Developing and Adult Brain International *Journal of Tryptophan Research* 3:141–149

Kanai, M., Funakoshi, H., Takahashi, H., Hayakawa, T., Mizuno, S., Matsumoto, K. and Nakamura, T. 2009. Tryptophan 2, 3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behaviour in mice. *Mol. Brain* 2 (8): 1-16

Kaper, T., Looger, L. L, Takanaga, H., Platten, M., Steinman, L., Frommer, W. B. 2007. Nanosensor detection of an immunoregulatory tryptophan influx/kynurenine efflux cycle. *PLoS Biol*. 5: e257.

Kee, N., Sivalingam, S., Boonstra R., Wojtowicz J.M. 2002. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J. *Neuroscience Methods* 115:97-105

Kerr, J. F., Wyllie, A. H., and Currie, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br .J. Cancer* 26,239–257.doi:10.1038/bjc.1972.33

Khaliq, S., Haider, S., Ahmed, S.P., Perveen, T., and Haleem D.J. 2006. Relatioship of brain Tryptophan and serotonin n improving cognitive performance in rats. *Pak. J. Pharm. Sci*., 19 (1): 11-15

Kiely, M. and Sourkes, T. L. 1972. Transport of L- tryptophan into slices of rat cerebral cortex. *J. Neurochem.*, 19, 2863-2872

Klomp, A., Va'clavu, L., Meerhoff, G. F., Reneman, L., Lucassen, P. J. 2015. Effects of chronic Fluoxetine treatment on neurogenesis and tryptophan hydroxylase expression in adolescent and adult rats. *PLoS ONE* 10(8): e0135876. doi:10.1371/ journal.pone 0135876

Knott, P.J. and Curzon, G. 1972. Free tryptophan in plasma and brain tryptophan metabolism. *Nature*, 239, 452-453.

Knox, W. E. and Greengard, O. 1964. Regulation of some enzymes of nitrogen metabolism. An introduction to enzyme physiology. In advances in enzyme regulation. G. Weber ed. New York: Pergamon. Vol. 3: 247.

Knusel, B., Michel, P. P., Schwaber, J. S. and Hefti, F. 1990. Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and insulin-like growth factor I and II. *J.Neurosci*. 10(2): 558-70

Kodama, M., Fujioka, T., Duman R. S. 2004. Chronic olanzapine or fluoxetine administration increases cell proliferation in hippocampus and prefrontal cortex of adult rat. *Biol Psychiatry* 56: 570-580.

Koirala, S., Corfas, G. 2010. Identification of novel glial genes by single-cell transcriptional profiling of Bergmann glial cells from mouse cerebellum. *PLoS One* 5:e9198.

Komine, O., Nagaoka, M., Watase, K., Gutmann, D.H., Tanigaki, K., Honjo, T., Radtke, F., Saito, T., Chiba, S., Tanaka, K. 2007. The monolayer formation of Bergmann glial cells is regulated by Notch / RBP-J signaling. *Developmental Biology* 311:238-250

Komuro, H., and Rakic, P. 1996. Intracellular Ca^{2+} fluctuations modulate the rate of neuronal migration. *Neuron* 17, 275–285.doi:10.1016 / s0896-6273(00) 80159-2

Komuro, H., Yacubova, E., Rakic, P. 2001. Mode and tempo of tangential cell migration in the cerebellar external granular layer. *J Neurosci*, 21:527-540.

Komuro, H.; Yacubova, E. 2003. Recent advances in cerebellar granule cell migration. Cell. *Mol. Life Sci.* 60, 1084–1098.

Komuro, Y., Kumada, T., Ohno, N., Foote, K. D., Komuro, H. 2013. Migration in the Cerebellum. In Cellular Migration and Formation of Neuronal Connections, 1st ed.; Rubenstein, J., Rakic, P., Eds.; Elsevier Inc.: Oxford, UK, 2013; Volume 2: 281–297.

Kool, M., Koster, J., Bunt, J., Hasselt, N.E., Lakeman, A., van Sluis, P., Troost, D., Meeteren, N. S, Caron, H. N., Cloos, J., Mrsic,´ A., Ylstra, B., Grajkowska, W., Hartmann, W., Pietsch, T., Ellison, D., Clifford, S.C., Versteeg, R. 2008. Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features. *PLoS ONE* 3:e3088.

Koppel, H., Lewis, P. D. and Wigglesworth, J.S. 1982. A study of the vascular supply to the external granular layer of the postnatal rat cerebellum *J. Anat.* 134, 1, pp. 73-84

Korsmeyer, S. J. 1993. Programmed cell death: *Bcl-2*. In: Important advances in oncology (DeVita VT, Helman S, Rosenberg SA, eds), pp 19–28. Philadelphia: Lippincott.

Kudo, Y., and Boyd C. A. R. 2001. Characterization of L-Tryptophan transporters in human placenta: a comparison of brush border and basal membrane vesicles. *Journal of Physiology* 531: 405-416

Laffaire, J., Rivals, I., Dauphinot, L., Pasteau, F., Wehrle, R., Larrat, B., Vitalis, T., Moldrich, R.X., Rossier, J., Sinkus, R., Herault, Y., Dusart, I., and Potier, M.C. 2009. Gene expression signature of cerebellar hypoplasia in a mouse model of Down syndrome during postnatal develoment. *BMC Genomics,* 10: 138 doi.org/10.1186/1471-2164-10-138

Lauder, J. M., Wallace, J. A., Wilkie, M. B., Krebs, H., 1983. Roles for serotonin in neurogenesis. Monogr. *Neural Sci.* 9, 3–10.

Le Floc'h N, Melchior D, Seve B. 2008. Dietary tryptophan helps to preserve tryptophan homeostasis in pigs suffering from lung inflammation. *Journal of Animal Science* 86:3473-3479

Leto, K., Arancillo, M., Becker, E., Buffo, A., Chiang, C., Ding, B., Dobyns, W. B. and Dusart, I. 2016. Consensus Paper: Cerebellar Development. *Cerebellum 15:*789-828

Levitsky, D. A., Strump, B. J., 1995. Malnutrition and the brain:changing concepts, changing concerns. *J. Nutr.* 125, 2212S–2220S.

Levitt, P., and Rakic P. 1980. Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J. Comp. Neurol.* 193: 815-840.

Lewis, P. D. 1975. Cell death in the germinal layers of the postnatal rat brain. *Neuropathol. Appl. Neurobiol.*1, 21–29.

Li, K., Leung, A. W., Guo, Q., Yang, W., and Li, J. Y. H. 2014. Shp2-Dependent ERK signaling is essential for induction of Bergmann glia and foliation of the cerebellum. *The Journal of Neuroscience,* 34 (3): 922-31

Liang, Y. D., Song, J. F. 2005. Flow-injection chemiluminescence determination of tryptophan through its peroxidation and epoxidation by peroxynitrous acid, *J. Pharm. Biomed. Anal. 38:*100–106.

Lin, X., Bulleit, R. F.1997. Insulin-like growth factor-I (IGF-I) is a critical trophic factor for developing cerebellar granule cells. Brain Res *Dev. Brain Res* 99:234-242

Lin, J. C., Cepko, C. L. 1998. Granule cell raphes and parasagittal domains of Purkinje cells: complementary patterns in the developing chick cerebellum. *J. Neurosci.* 18:9342–9353.

Lieben, C. K., Van O, Deutz, N. and Blokland, A. 2004. Acute tryptophan depletion induced by a gelatin based mixture impairs object memory but not effective behavior and spatial learning in rats. *Behav. Brain Res.*, 151(1-2): 53-64.

Linas, R. R., Walton, K. D, Lang, E. J. 2004. "Cerebellum". The Synaptic Organization of the Brain. New York: Oxford University Press. Ch. 7.

Linseman, D.A., Phelps, R.A., Bouchard, R.J., Le Tracey, S.S., Laessig, A., McClure, M, L and Heidenreich, K. A. 2002. Insulin-like growth factor I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons. *The Journal of Neuroscience,* 22 (21):9287-97

Lossi, L., Coli, E., Gianessi, E., Stornelli, M. R., Marroni, P. 2002. Cell proliferation and apoptosis during histogenesis of the guinea pig and rabbit cerebellar cortex. *Italian Journal of Anatomy and Embryology* 107(2):117-125

Lossi, L. 2004. Occurrence of two different mechanisms of apoptosis in cerebellar granule cells in relation to the specificity of poly-ADP-ribosepolymerase- 1 (PARP) activation. *Vet. Res. Commun.* 28 (Suppl.1), 197–200 .doi:10. 1023/b:verc.0000045405.66323.4d

Lucassen, P. J., Meerlo, P., Naylor, A.S., van Dam, A. M., Dayer, A. G., Fuchs, E., Oomen, C. A. Czeh, B. 2010. Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. *Eur. Neuropsychopharmacol.* 20(1):1-17.

Ma, S., Kwon, H. J., Huang, Z. 2012. Ric-8a, a guanine nucleotide exchange factor for hetertrimeric G proteins regulates Bergmann glia-basement membrane adhesion during cerebellar foliation. *J. Neurosci* 32:14979-993

MacCallum, D. E., Hall, P. A. 2000. The biochemical characterization of the DNA binding activity of pKi67. *The Journal of Pathology* 191:286–298.

Malberg, J. E., Eisch, A. J., Nestler, E. J., Duman, R. S. 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus *J. Neuroscience.* 20: 9104-9110

Marazziti, D., Di Pietro, C., Golini, E., Mandillo, S., La Sala, G., Matteoni, R and Tocchini-Valentini, G.P. 2013. Precocious cerebellum development and improved motor functions in mice lacking the astrocyte cilium-patched 1-associated Gpr37l1 receptor *PNAS* Vol. 110(41):16486–16491

Mares, V., Lodin, Z. 1970. The cellular kinetics of the developing mouse cerebellum. II. The function of the external granular layer in the process of gyrification. *Brain Res*, 23:343-352.

Maricich, S.M., Herrup, K. 1999. Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. *J. Neurobiol.* 41, 281–294.

Marlatt, M. W., Potter, M. C., Bayer, T. A., van Praag, H., Lucassen, P. J. 2013. Prolonged running, not fluoxetine treatment increases neurogenesis but does not alter neurppathology in the 3xTg mouse model of Alzheimer's disease. Curr. Top. *Behav. Neurosci.* 15: 313-340

Marzban, H., Del Bigio, M.R., Alizadeh, J., Ghavami, S., Zachariah, R.M. and Rastegar, M. 2015. Cellular commitment in the developing cerebellum *Frontiers in cellular neuroscience* Vol 8 Article 450: 1-26

Marzban, H., Hoy, N., Buchok, M., Catania, K. C., and Hawkes, R. 2015. Compartmentation of the cerebellar cortex: adaptation to lifestyle in the star- nosed mole Condylura cristata. *Cerebellum* 14:106-118.

Mathews, L. S., Hammer, R. E., Behringer, R. R., D'Ercole, A..J., Bell, G. I., Brinster, R. L., Palmiter, R.D. 1988. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* 123:2827-33

McConnell, S. K., Kaznowski, C. E. 1991. Cell cycle dependence of laminar determination in developing neocortex. *Science* 254:282–285.

Merry, D. E., Veis, D. J., Hickey, W. F., and Korsmeyer, S. J. 1994. Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* 120:301–311.

Miale, I. L., Sidman, R. L. 1961. An autoradiographic analysis of histogenesis of the mouse cerebellum. *Exp. Neurol* 4:277–296.

Mizuseki, K., Sakamoto, T., Watanabe, K., Muguruma, K, Ikeya, M, Nishiyama, A. Arakawa A. Suemori, H., Nakatsuji, N, Kawasaki, H., Murakami, F., Sasai, Y. 2003. Generation of neural crest –derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. *Proc. Natl. Acad. Sci.* USA. 100, 5828-33

Moffat, A. C., Jackson, J. V., Moss, M. S., Widdop, B. 1986. Clarke's Isolation and Identification of Drugs. The Pharmaceutical Press,London, UK p. 1056.

Morin, F., Diño, M. R., Mugnaini, E. 2001. Postnatal differentiation of unipolar brush cells and mossy fiber-unipolar brush cell synapses in rat cerebellum. *Neuroscience*. 104:1127–39.

Moroni F. 1999. Tryptophan metabolism and brain function: focus on kynurenine and other indole metabolites. *Eur J Pharmacology*. 375(1–3):87–100.

Mugnaini, E., Sekerková, G., Martina, M. 2011. The unipolar brush cell: a remarkable neuron finally receiving deserved attention. *Brain Res Rev.* 66:220–45.

Muller, Y., Tangre, K., Clos, J. 1997. Autocrine regulation of apoptosis and bcl-2 expression by nerve growth factor in early differentiating cerebellar granule neurons involves low affinity neurotrophin receptor. *Neurochem Int* 31:177–191.

Murthi, P., Stacey E., Dickinson, H., Euan, D. W., Wallace, P. E. 2016. Placental tryptophan metabolism and serotonin transport are distrupted in idiopathic human fetal growth restriction. Abstracts/ *Placenta* 45: 63-133

Nagata, I., Nakatsuji, N. 1990. Granule cell behavior on laminin in cerebellar microexplant cultures. *Dev Brain Res* 52:63–73.

Nakatsuji, N., Nagata, I. 1989. Paradoxical perpendicular contact guidance displayed by mouse cerebellar granule cell neurons in vitro. *Development* 106, 441-47

Nunzi, M. G., Mugnaini, E. 2000. Unipolar brush cell axons form a large system of intrinsic mossy fibers in the postnatal vestibulocerebellum. *J Comp Neurol.* 422:55–65.

O'Kusky, J. R., Ye, P. and D'Ercole, A. J. 2000. Insulin-like growth factor - I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *Journal of Neuroscience*, 20(22): 8435-42.

O'Rourke, N. A., Chenn, A., McConnell S. K. 1997. Postmitotic neurons migrate tangentially in the cortical ventricular zone. *Development* 124:997–1005.

Obersteiner, H. 1883. Der Feinere bau der Kleinhirnrinde bein Menschen und bei Tiercn. *Biol. Zentralbl.*3: 145- 155.

Ogungbenro, K., Aarons, L. and CRESim & Epi-CRESim Project Groups. 2014. Physiologically based pharmacokinetic modeling of methotrexate and 6-mercaptopurine in adults and children. Part 1: methotrexate. *J. Pharmacokinet. Pharmacodyn*. 41: 159-171.

Okano-Uchida, T., Himi, T., Komiya, Y., and Ishizaki, Y. 2004. Cerebellar granule cell precursors can differentiate into astroglial cells. *Proc. Natl. Acad .Sci. USA* 101, 1211–1216.

Oldendorf, W.H. 1971. Brain uptake of radiolabelled amino acids, amines and hexoses after arterial injection. *Amer. J. Physiol.,* 221, 1629-1639.

Ono, K., Kawamura, K. 1990. Mode of neuronal migration in the pontine stream of fetal mice. *Anat. Embryol.* 182:11-19

Oppenheim, R. W. 1991. Cell death during development of the nervous system. Annu. Rev. *Neurosci.*14, 453–501.

Pai'zanis, E., Renoir, T., Lelievre, V., Saurini, F., Melfort, M., Gabriel, C., Barden, N., Mocaer. E., Hamon, M., Lanfumey, L., 2010. Behavioural and neuroplastic effects of the new-generation antidepressant agomelatine compared to fluoxetine in glucocorticoid receptor-impaired mice. *Int. J. Neuropsychopharmacol.* 13 (6): 759-774.

Palay, S.L., McGee-Russell, S.M., Gordon, S. and Grillo, M. A. 1962. Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide. *J. Cell Biol.* 12(2):385-410

Palkovitz, M., Magyar, P. and Szentagothai J. 1971a. Quantitative histological analysis of the cerebellar cortex in cat II. Cell numbers and density in the granular layer. *Brain Res*., 32:15-30

Palkovitz, M., Magyar, P. and Szentagothai J. 1971b. Quantitative histological analysis of the cerebellar cortex in cat III. Structural organization of the molecular layer. *Brain Res.* 34:1-18

Peters, J. C. 1991. Tryptophan nutrition and metabolism: an overview. *Adv. Exp. Med. Biol*. 294:345–58.

Ponce, R. A., Kavanagh, T. J., Mottet, N. K., Whittaker, S. G., Faustman, E. M. 1994. Effects of methyl mercury on the cell cycle of primary rat CNS cells in vitro. *Toxicol Appl Pharmacol* 127:83–90.

Prescott, J. W. 1997. Touch, The Future Newsletter, Spring 1997

Purves, D., Augustine, G. J., Fitzpatrick, D., Hall, W. C., LaMantia, A., McNamara J. O., and White, L. E. 2008. *Neuroscience.* 4th ed. Sinauer Associates. pp. 432–4.

Raaf, J., Kernohan, J.W. 1944. A study of the external granular layer in the cerebellum. The disappearance of the external granular layer and the growth of the molecular and intemal granular layer in the cerebellum. *Am. J. Anat*. 75, 151-172

Rabié, A., Favre, C., Clavel, M. C., and Legrand, J. 1977. Effects of thyroid dysfunction on the development of the rat cerebellum, with special reference to cell death within the internal granular layer. *Brain Res.*120, 521–531.

Rahmanzadeh, R., Hüttmann, G., Gerdes, J., Scholzen, T. 2007. "Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis". *Cell Prolif.*40 (3): 422–30.

Rakic, P. 1971. Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in *Macacus* rhesus. *J Comp Neurol*. 141:283–312.

Rakic, P. 1985. Mechanisms of neuronal migration in developing cerebellar cortex. In: Molecular basis of neural development (Edelman G.E., Cowan, W.M., Gall, E. eds) pp 139-160 New York: Wiley

Rakic, P., Cameron, R. S., Komuro, H. 1994. Recognition, adhesion, transmembrane signaling and cell motility in guided neuronal migration. *Curr Opin Neurobiol* 4:63– 69.

Rakic, P., Knyihar-Csillik, E. and Csillik, B. 1996. Polarity of microtubule assembly during neuronal migration. *Proc. Natl Acad Sci* USA 93:9218-9222

Ramon y, Cajal S. 1911. Histologie du Systeme Nerveux de l'Homme et des Vertebres Volume 2. Edited by: Azoulay L. Paris: Maloine. 2,153-173

Ramos- Vara, J. A. 2011. Principles and methods of immunohistochemistry. *Methods Mol. Biol* 691:83-96

Raoult, E.; Bénard, M.; Komuro, H.; Lebon, A.; Vivien, D.; Fournier, A.; Vaudry, H.; Vaudry, D.; Galas, L. 2014. Cortical-layer-specific effects of PACAP and tPA on interneuron migration during post-natal development of the cerebellum. *J. Neurochem.* 130, 241–254.

Reznikov, K. Y. 1991. Cell proliferation and cytogenesis in the mouse hippocampus. *Advances in Anatomy, Embryology and Cell Biology* Vol 122:19-32

Rivas, R. J., Hatten, M. E. 1995. Motility and cytoskeletal organization of migrating cerebellar granule neurons. *J Neurosci* 15:981–989.

Rivest, R. W. 1991. Sexual maturation in female rats: Heredity, developmental and environmental aspects. *Experientia* 47, 1026– 1038.

Roper, R. J., Baxter, L. L., Saran, N. G., Klinedinst, D. K., Beachy, P. A., Reeves, R. H. 2006. Defective cerebellar response to mitogenic Hedgehog signaling in Down syndrome mice. *PNAS.* 103 (5): 1452-6

Rossi, F., Corvetti, L., Gianola, S. 2006. The strange case of Purkinje axon regeneration and plasticity. *Cerebellum.* 5:163–73.

Ruan Z., Yang Y. H., Wen, Y. M., Zhou, Y., Fu, X. F., Liu, G., Yao, K., Wu, X., Deng, Z. Y., Wu, G.Y., Yin, Y., L.2014. Metabolomic analysis of amino acid and fat metabolism in rats with Tryptophan supplementation *Amino acids* 46:2681-2681

Ruddick, J. P., Evans, A. K., Nutt, D. J., Lightman, S. L., Rook, G. A., Lowry, C. A. 2006. Tryptophan metabolism in the central nervous system: medical implication. *Expert. Rev. Mol. Med.* 8(20):1-27

Russell, J. W., Windebank, A. J., Schenone, A., Feldman, E. L. 1998. Insulin-like growth factor-I prevents apoptosis in neurons after nerve growth factor withdrawal. *J. Neurobiol.* 36:455-467

Ryder, E. F., Cepko, C. L. 1994. Migration patterns of clonally related granule cells and their progenitors in the developing chick cerebellum. *Neuron* 12:1011–1029.

Sadler, T. W. 2012. Langman's Medical Embryology. 12th Ed. Philadelphia: Wolters Kluwer Health/ Lippincott Williama and Wilkins pg 452

Salas, S. P., Giacaman, A., Romero, W., Downey, P., Aranda, E., Mezzano, D., Vio, C. P. 2007. Pregnant rats treated with a serotonin precursor have reduced fetal weight and lower plasma volume and kallikrein levels. *Hypertension*50:773-779.

Santana, C. Martin, L., Valladares, F., Diaz-Flores, L., Santana-Herrera, C., Milena, A. and Diaz M. R. 1999. Tryptophan ingestion by pregnant rat induces pituitary and mammary tumours in the adult female offspring. *Hum Reprod.* Vol. 14, (8) 2155-2161

Sato, N., Hotta, K., Waguri, S., Nitatori, T., Tohyama, K., Tsujimoto, Y., Uchiyama, Y. 1994. Neuronal differentiation of PC12 cells as a result of prevention of cell death by bcl-2. *J Neurobiol* 25:1227–1234.

Scherini, E. and Bernocchi, G. 1994. *Cis* DDP treatment and development of the rat cerebellum. *Prog. Neurobiol.* 42:161–196.

Schroecksnadel, K., Wirleitner, B., Winkler, C., Fuchs, D. 2006. Monitoring tryptophan metabolism in chronic immune activation. *Clin Chim Acta*. 364:82–90. [PubMed: 16139256]

Schroeder, W. A., Kay, L. M., Mills, R. S. 1990. Spectrophotometric analysis of amino acids using ninhydrin chemical reaction. Anal. Chem., 22:760

Segal, R. A., Takahashi, H. and Mckay, R.D.G. 1992. Changes in neurotrophin responsiveness during the development of the cerebellar granule neurons. *Neuron.* 9, 1041-52.

Segall, P. E. and Timiras P. S. 1976. Pathophysiologic findings after chronic tryptophan deficiency in rats: a model for delayed growth and aging. *Mechanisms of Ageing and Development* Vol 5:109-124

Sekerková, G., Ilijic, E., Mugnaini, E. 2004. Time of origin of unipolar brush cells in the rat cerebellum as observed by prenatal bromodeoxyuridine labeling. *Neuroscience.* 127:845–58.

Shambaugh, G. E., Lee, R. J., Watanabe, G., Erfurth, F., Karnezis, A. N., Koch, A. E., Haines, G. K., Halloram, M., Brody, B. A., Pestell, R. G. 1996. Reduced cyclin D1 expression in the cerebella of nutrition-deprived rats correlates with development delay anddecreased cellular DNA synthesis. *J. Neuropathol. Exp. Neurol.* 55, 1009 – 1020.

Shiga, T., Ichikawa, M., Hirata, Y., 1983. A Golgi study of Bergmann glial cells in developing rat cerebellum. *Anat. Embryol.* 167, 191–201.

Silbereis, J., Cheng, E., Ganat, Y. M., Ment, L. R., Vaccarino, F. M. 2009. Precursors with glial fibrillary acidic protein promoter activity transiently generate GABA interneurons in the postnatal cerebellum. *Stem Cells.* 27:1152–63.

Silbereis, J., Heintz, T., Taylor, M. M., Ganat, Y., Ment, L.R., Bordey, A., Vaccarino, F. 2010. Astroglial cells in the external granular layer are precursors of cerebellar granule neurons in neonates. *Mol. Cell Neurosci.* 44(4):362-73.

Sillitoe, R. V., and Joyner, A. L. 2007. Morphology, molecular codes andcircuitry produce the three-dimensional complexity of the cerebellum. *Annu. Rev. Cell Dev. Biol.*23, 549–577.

Smart, I. H. M. 1982. Radial unit analysis of hippocampal histogenesis in the mouse. *J. Anat.* 135:763–93

Smeyne, R. J. and Goldwitz, D. 1989. Development and death of external granular layer cells in the weaver mouse cerebellum, a quantitative study. *The Journal of Neuroscience,* 9(5): 1608-20

Smith, Q. R. S., Stoll, J. 1998. Blood–brain barrier amino acid transport. In: Pardridge WM (ed) Introduction to the blood–brain-barrier. University Press, Cambridge, pp 189–197

Smith, Q. R., Momma, S., Aoyagi, M., Rapoport, S. I. 1987a. Kinetics of neutral amino acid transport across the blood–brain barrier. *J Neurochem* 49(5):1651–1658

Sommer, I., Lagenaur, C., Schachner, M. 1981. Recognition of Bergmann glial and ependymal cells in the mouse nervous system by monoclonal antibody. *J. Cell Biol.* 90(2):448-458

Sotelo, C. 1990. Cerebellar synaptogenesis: what we can learn from mutant mice. *J. Exp. Biol*. 153:225-249.

Sotelo, C., Alvarado-Mallart R. M., Frain, M., Vernet, M. 1994. Molecular plasticity of adult Bergmann fibers is associated with radial migration of grafted Purkinje cells. *Journal of Neuroscience* 14, 124-133.

Sotelo, C. 2004. Cellular and genetic regulation of the development of the cerebellar system. *Prog. Neurobiol.* 72, 295-339.

Soto-Moyano, R., Alarco´ n, S., Herna´ndez, A., Pe´rez, H., Ruı´z, S., Carren˜ O, P., Kusch, C., Belmar, J. 1998. Prenatal malnutrition induced functional alterations in callosal connections and in interhemispheric asymmetry in rats are prevented by reduction of noradrenaline synthesis during gestation. *J. Nutr.* 128, 1224– 1231.

Su, H., Muguruma, K., Matsuo-Takasaki, M., Kengaku, M., Watanabe, K., Sasai, Y. 2006. Generation of cerebellar neuron precursors from embryonic stem cells. *Developmental Biology* 290(2); 287 – 296

Sudarov, A., Turnbull, R. K., Kim, E. J., Lebel-Potter, M., Guillemot, F., Joyner, A. L. 2011. Ascl1 genetics reveals insights into cerebellum local circuit assembly. *J. Neurosci.* 31:11055–69.

Sugiyama, A., Sun, J., Ueda, K., Furukawa, S. and Takeuchi, T. 2015. Effect of methotrexate on cerebellar development in infants rats. Jvms. 14-0475: *J. Vet. Med. Sci* 77(7) 789-797

Tagakami, M., Yamagata, K, Nara, Y, Fujino, H, Kubota, A., Numano, F., Yamori, Y. 1997. Insulin-like growth factors prevent apoptosis in cortical neurons isolated from stroke-prone spontaneously hypertensive rats. *Lab Invest.* 76:603-612

Tagliamonte, A., Tagliamonte, P., Forn, J., Perezcruet, J., Krishna, G. and Gessa, G. L. 1971. Stimulation of brain serotonin synthesis by dibutyrylcyclic AMP in rats. *J. Neurochem.*, 18, 1191-1196.

Taroni, F., DiDonato, S. 2004. Pathways to motor incoordination: the inherited ataxias. Nat. Rev., *Neurosci.* 5, 641-55

Tavano, A., Grasso, R., Gagliardi, C., Triuzli, F., Bresolin, N., Fabbro, F., Borgatti, R. 2007. Disorders of cognitive and affective development incerebellar malformations. *Brain,*130:2646-2660.

ten Donkelaar, H. J., M. Lammens, P. Wesseling, H. O. Thijssen, and W. O. Renier. 2003. Development and developmental disorders of the human cerebellum.*J. Neurol.*250:1025–1036.

Tone, S., Takikawa, O., Habara-Ohkubo, A., Kadoya, A., Yoshida, R., Kido, R. 1990. Primary structure of human indoleamine 2, 3-dioxygenase deduced from the nucleotide sequence of its cDNA. *Nucleic Acids Res*. 18:367.

Trenkner, E., Smith D. and Segil, N. 1984. Is cerebellar granule cell migration regulated by an internal clock? *The Journal of Neuroscience* vol 4, No 11: 2850 – 2855

Trinkhaus, J. P. 1984 Cells into Organs. The Forces that Shape the Embryo, 2nd ed., Prentice-Hall, Englewood Cliffs, NJ.

Tyrrell, T., and Willshaw, D. 1992. "Cerebellar cortex: its simulation and the relevance of Marr's theory". Philosophical Transactions of the Royal Society of London. Series B, *Biological Sciences.* 336 (1277): 239–57.

Van Den Berg, R and van Der Eecken, H. 1968. Anatomy and embryology of cerebral circulation. *Progress in Brain Research.* Vol.30:1-25 (Ed. W. Lugendijk) Amsterdam, Elsevier

van Dorp, S., De Zeeuw, C. I. 2014. Variable timing of synaptic transmission in cerebellar unipolar brush cells. *Proc Natl Acad Sci* U S A. 111:5403–8.

Voogd, J. 2011. Cerebellar zones: a personal history. *Cerebellum* 10, 334–350.

Wadiche, J. I. and Jahr, C. E. 2001. "Multivesicular release at climbing fiber-Purkinje cell synapses". *Neuron.*32 (2): 301–13.

Wechsler-Reya, R. J, Scott, M. P. 1999. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* 22:103–114.

Wechsler-Reya, R., Scott M. P. 2001. The developmental biology of brain tumors. *Annu. Rev.Neurosci*. 24:385–428.

Welker, W. I. 1990. The significance of foliation and fissuration of cerebellar cortex. The cerebellar folium as a fundamental unit of sensorimotor integration. *Arch Ital Biol*128:87-109.

Werther, G.A., Cheesman, H., Russo, V. 1993. Olfactory bulb organ culture is supported by combined insulin- like growth factor-I and basic fibroblast growth factor. *Brain Res* 617:339-42

Wichterle, H., Garcia-Verdugo, J. M., Alvarez-Buylla, A. 1997. Direct evidence for homotypic, glia-independent neuronal migration. *Neuron* 18:779 –791.

Williams, G. T., Smith, C. A. 1993. Molecular regulation of apoptosis: genetic controls on cell death. *Cell* 74:777–779.

Wong, R. O. L., and Hughes, A. 1987. Role of cell death in the topogenesis of neuronal distributions in the developing cat retinal ganglion cell layer. *J. Comp. Neurol.* 262: 496-5 11.

Wood, K. A., Dipasquale, B., Youle, R. J., 1993. In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron* 11: 621– 632.

Woodger, T. L., Sirek, A., Anderson, G. H. 1979. Diabetes, dietary tryptophan, and protein-intake regulation in weanling rats. *The American Journal of Physiology* 236:R307-R311

Woolley III, P. V., Dion, R. L and Bono, Jr. V. H. 1974. Effects of Tryptophan Deprivation on L1210 Cells in Culture *Cancer Res* 34:1010-1014.

Wulliman, M. F., Mueller, T., Distel, M., Babaryka, A., Grothe, B. and Koster R. W. 2011. The long adventurous journey of rhombic lip cells in jawed vertebrate: a comparative developmental analysis. *Front. Neuroanat.* 5:27

www. Ratpix.pdf 2008

www.alz forum.org

www.sciencedirect.org

www.sciencemag.org

www.sciencemag.org

www.semantics scholar.com

Wyllie, A. H., Kerr, J. F. R. and Currie A. R. 1980. Cell death: The significance of apoptosis. *Int. Rev. Cytol.* 68: 25l-306.

Xu, H., Yang, Y., Tang, X., Zhao, M., Liang, F., Xu, P., Hou, B., Xing, Y., Bao, X., Fan, X. 2013. Bergmann glial function in granule cell migration during cerebellum development. *Mol. Neurobiol.* 47:833-44

Yamada, K., Fukaya, M., Shibata, T., Kurihara, H., Tanaka, K, Inoue, Y, Watanabe, M. 2000. Dynamic transformation of Bergmann glial fibers proceeds in correlation with dendritic outgrowth and synapse formation of cerebellar Purkinje cells. *J. Comp. Neurol.* 418, 106-120.

Yamada, K., Watanabe, M. 2002. Cytodifferentiation of Bergmann glia and its relationship with Purkinje cells. *Anat. Sci. Int.* 77:94-108

Yamanaka, H., Yanagawa, Y., and Obata, K. 2004. Development of stellate and basket cells and their apoptosis in mouse cerebellar cortex. *Neurosci. Res*. 59, 13-22

Yamasaki, T., Kawaji, K., Ono, K., Bito, H., Hirani, T., Osumi, N., Kengaku, M. 2001. Pax6 regulates granule cell polarization during parallel fiber formation in the developing cerebellum. *Development*. 128: 3133-44.

Ye, P., Xing, Y., Dai, Z., D'Ercole, A. J. 1996. *In vivo* actions of insulin-like growth factor-I (IGF-I) on cerebellum development in transgenic mice: evidence that IGF-I increases proliferation of granule cell progenitors. *Dev. Brain Res* 95:44-54

Yuasa, S.; Kawamura, K.; Ono, K.; Yamakuni, T.; Takahashi, Y. 1991. Development and migration of Purkinje cells in the mouse cerebellar primordium. *Anat. Embryol.* 184, 195–212.

Yuasa, S., 1996. Bergmann glial development in the mouse cerebellum as revealed by tenascin expression. *Anat Embryol.* 194:223– 34.

Zanjani, H. S., Vogel, M. W., Delhaye-Bouchaud, N., Martinou, J. C., Mariani, J. 1996. Increased cerebellar Purkinje cell numbers in mice overexpressing a human bcl-2 transgene. *J. Comp. Neurol.* 374:332–341.

Zanjani, H. S., Vogel, M. W., Delhaye-Bouchaud, N., Martinou, J. C., Mariani, J. 1997. Increased olivary neuron and cerebellar granule cell numbers in transgenic mice overexpressing the human *bcl-2* gene. *J Neurobiol* 32:502–516.

Zanjani, H. S., Vogel, M.W., Martinou, J. C., Delhaye-Bouchaud, N and Mariani, J. 1998. Postnatal Expression of *Hu-Bcl-2* Gene in *Lurcher* Mutant Mice Fails to Rescue Purkinje Cells but Protects Inferior Olivary Neurons from Target-Related Cell Death. *The Journal of Neuroscience*, *18*(1):319–327

Zecevic, N., Rakic, P. 1976. Differentiation of Purkinje cells and their relationship to other components of developing cerebellar cortex in man. *J. Comp Neurol* l67:27– 48.

Zhang, L., Goldman, J. E. 1996. Developmental fates and migratory pathways of dividing progenitors in the postnatal rat cerebellum. *J. Comp. Neurol.* 370, 536- 550.

Zhang, K. and Sejnowski, T. J. 2000. A un iversal scaling law between gray matter and white matter of cerebral cortex. *Proc. Natl. Acad. Sci. USA.* 97, 5621-26

Zhang, C., Hua, T., Zhu, Z., and Xun L. X. 2006. Age-related changes of structures in cerebellar cortex of cat *J. Biosci.* 31(1) 55–60,Indian Academy of Sciences

Zheng, X., Zugates, C. T., Lu, Z., Shi, L., Bai., J. M., Lee, T. 2006. Baboon/dSmad2 TGF-beta signaling ie required during late larval stage for development of adult-specific neurons. *EMBO J.* 25(3):615-627

Zheng, J. N, Pei, D. S, Mao, L. J., Liu, X.Y., Mei, D. D., Zhang, B. F., Shi, Z., Wen, R. M, Sun, X. Q. 2009. Inhibition of renal cancer cell growth in vitro and in vivo with oncolytic adenovirus armed short hairpin RNA targeting Ki-67 encoding mRNA. *Cancer Gene Therapy* 16:20–32.

Zhong, L. T., Sarafian, T., Kane, D. J., Charles, A. C, Mah, S.P., Edwards, R. H., Bredesen, D. E. 1993. Bcl-2 inhibits death of central neural cells induced by multiple agents. *Proc Natl Acad Sci USA* 90:4533– 4537.

APPENDICES

TABLES

ONE WAY ANOVA

Tukey's Comparison of Serum Percentage of all groups

PND 7	PND 14	PND 21
0.0820	0.3817	0.0393

Tukey's Comparison of Brain weight of all groups

PND 0	PND 7	PND 14	PND 21
P<0.0001	0.0027	0.4662	0.0049

Tukey's Comparison of Cerebellar weight of all groups

PND 0	PND 7	PND 14	PND 21
0.0143	P<0.0001	P<0.0001	P<0.0001

Tukey's Comparison of EGL Thickness of all groups

PND 0	PND 7	PND 14	PND 21
0.1713	0.0054	0.1638	0.0031

Tukey's Comparison of EGL Density of all groups

PND 0	PND 7	PND 14	PND 21
P<0.0001	0.2134	P<0.0001	0.0006