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Internship Report

Study of cerebellum development in snakes

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From 10/06/2019 To 19/07/2019

At King's College London in the Developmental Neurobiology Department

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Acknowledgments

Firstly, I would like to communicate my special thanks of gratitude to Pr Richard Wingate, Head of Anatomy at King's College London, as well as Dr. Leigh Jane Wilson, who provided me the golden opportunity to do this wonderful internship on Developmental biology of Snakes cerebellum during embryogenesis. I am also thanking Vicky Rook, who overseed me during this 6 weeks internship and taught me many precise scientific techniques that will come in handy in the future.

Secondly, I want to thank my parents who financed my journey to London and helped me a lot in achieving this project within the limited time frame, and my friends who supported me during my first time living abroad alone experience.

STUDY OF CEREBELLUM DEVELOPMENT IN SNAKES

Études du développement du cérébellum chez les serpents

Abstract

The goal of my research project was to identify the 3-dimensional ordering of Purkinje cells flattened dendritic trees to see if their alignment allows the passage of parallel fibers in snakes cerebellum. I looked at the positioning of Purkinje cells in snakes brains. To do so, I performed three kinds of experiences: Nissl stains, Immunohistochemistry stains, and Golgi stains. Nissl stain showed that cell stratification of the snake's cerebellum is similar to those of other animals, except for the absence of a Purkinje layer. The snake cerebellar cortex contains a Molecular layer and a Nuclear layer. Immunohistochemistry showed that Purkinje cells were spread all through the Molecular layer. Flattened dendritic arbors do not face one another sticking flat together. Golgi stain even shows that they might be oriented in different dimensions. So they can not all be interconnected by parallel fibers from the deeper-layers. Therefore, they may not communicate with the rest of the body. We might speculate on an existing correlation between limbs and Purkinje cell repartition in vertebrates.

Abstract

Mon projet de recherche était d'identifier le placement en trois dimension des dendrites des cellules de Purkinje en regardant leur positionnement dans le cerebellum de diverses espèces de serpents. Pour cela j'ai réalisé 3 types de colorations: la coloration Golgi, Nissl et Immunohistochimie. Ces dernières ont montré que la stratification des cellules du cerebellum des serpents est similaire à celles de d'autres animaux, à l'exceptions que les serpents manque la couche de Purkinje. Immunohistochimie montre que les cellules de Purkinje sont dispersé dans la couche moléculaire. La coloration Golgi montre que les arborescences dendritiques des cellules de Purkinje semblent être toutes orientés dans des positions différentes. Ainsi les dendrites ne peuvent pas être interconnecté par des fibres parallèles. On peut spéculer sur l'existence d'une corrélation entre les membres et les cellules de Purkinje chez les vertébrés.

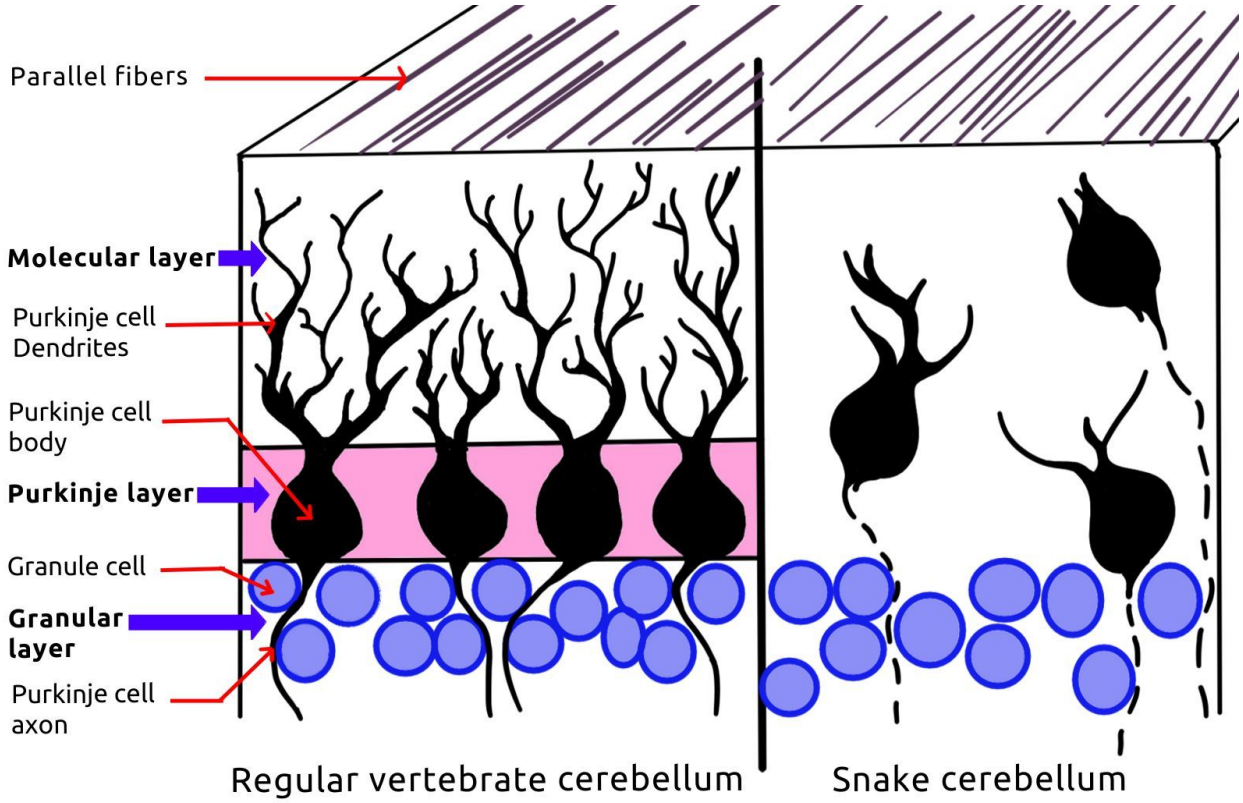
Keywords

Purkinje cells, Dendrites, Golgi stain, Nissl stain, Immunohistochemistry, snake's cerebellum

Mots clefs

Cellule de Purkinje, Dendrites, Coloration de Golgi, Coloration de Nissl, Immunohistochimie, cerebellum du serpent

Graphical abstract



Schematisation of cerebellum in most vertebrates and in snakes.

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Introduction

The cerebellum is part of the hindbrain of all vertebrates. It regulates motor movements and coordinates voluntary movements such as posture, balance, coordination, and speech, resulting in sleek and steady muscular activity.[1][2] It is also believed to take part in some cognitive functions such as attention, language, fear regulating and pleasure responses.[2][3] The cerebellum receives information from the sensory systems of the spinal cord and other parts of the brain.[1][2][4] It integrates these pieces of information to regulates motor activity and movements. Cerebellar injury causes disorders in movement, equilibrium, posture, and motor learning in humans.[2][4]

The cerebellum is made of a tightly folded layer of cortex called grey matter, with white matter underneath. There are two main kinds of neurons in the cerebellum: Purkinje cells, and Granule cells.[1] The cerebellar cortex is subdivided into three layers: the Granule layer also called Nuclear layer (dense, composed in majority of Granule cells), the Purkinje layer (composed by the cell bodies of Purkinje cells), and the Molecular layer (containing the flattened dendritic trees of Purkinje cells).[2][5] Purkinje cells are among the largest neurons in the human brain. They are constituted of a large and elaborate dendritic arbor spreading from the cell body (nuclei).[2][5] These dendritic trees lay so flat that they nearly form two-dimensional layers. Purkinje cells are aligned like dominos stacked one in front of the other. Dendritic arbors face one another and stick flat together like the pages of a book. A huge array of parallel fibers, which are the axons of granule cells, go through Purkinje cells large dendritic arbors at right angles. (Figure 1) There are up to 200 000 parallel fibers forming a Granule-cell-Purkinje-cell synapse with a single Purkinje cell.[2][5]

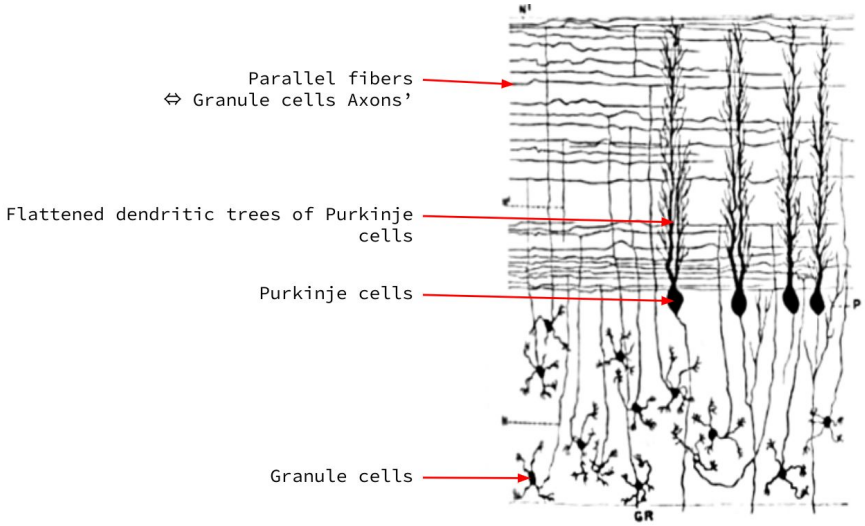


Figure 1: Scheme of Granule cells (GR, bottom), parallel fibers (horizontal lines, top), and Purkinje cells (P, middle) with flattened dendritic trees, modified from "Cerebellum." *Wikipedia*, November 30, 2019.[2]

The first Purkinje cells to develop shape the fourth ventricle, a thin layer of cells over a diamond-shaped cavity.[5] This ventricular zone in the neural tube forms the precursor of the nervous system in the embryo. Purkinje cells then migrate from there toward the outer surface of the cerebellar cortex, the cerebellum's center-lying section to be exact.[5] This allows the formation of the Purkinje cell layer. Purkinje cell development relies on a glycoprotein called Reelin, among several other proteins. Reelin helps to assemble Purkinje cells along a thick structure called the Purkinje plate and then along a single layer of cells in the cerebellum, the Purkinje cell layer.[5]

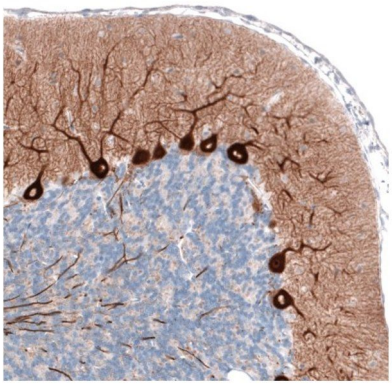


Figure 2: Immunohistochemistry staining of rat cerebellum using Mouse anti-Human PCP4 Antibody (CL5306). We clearly see ordered Purkinje cells (black-brown cells popping out), with their flat dendritic trees, and well-aligned cell bodies making the Purkinje layer. This section can be compared to a page of a book, with other quasi identical sections on each side of this particular one.
Taken from https://www.antibodies-online.com/ "Mouse Anti-Human PCP4 Antibody (ABIN5663260)." Accessed December 1, 2019.

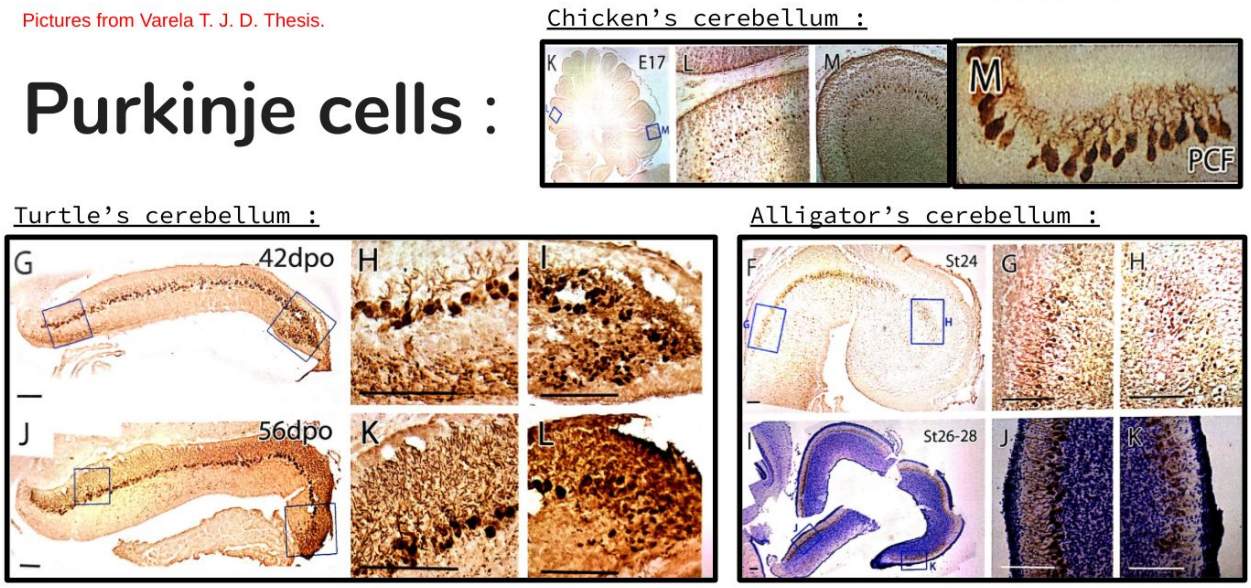


Figure 3: Immunohistochemistry staining of multiples animals cerebellum. Lhx1 in embryonic chicken cerebella, Calbindin in turtle and alligator cerebella. We see Purkinje cells (black-brown cells popping out) aligned forming the Purkinje layer, with their flat dendritic trees. Taken from Tristan Varela Thesis.[6]

Rats have well ordered Purkinje cells (Figure 2), but many other animals have to (Figure 3). Purkinje cell alignment seems to have a crucial impact on correct cerebellar development, and

healthy life. Reeler pathology affects the motricity. Reeler mouse, mouse affected by a spontaneous mutation affecting the correct expression of Reelin during the development of the central nervous system, showed difficulties moving around the cage.[2]

Looking at snakes cerebellum (Figure 4), Mr. Tristan Jan Dacles Varela, a former Ph.D. student at the Department of Developmental Neurobiology at King's College London, supervised by Richard Wingate, discovered that they do not possess a Purkinje layer. Snakes seem to have Purkinje cells scattered all throughout the molecular layer.[6]

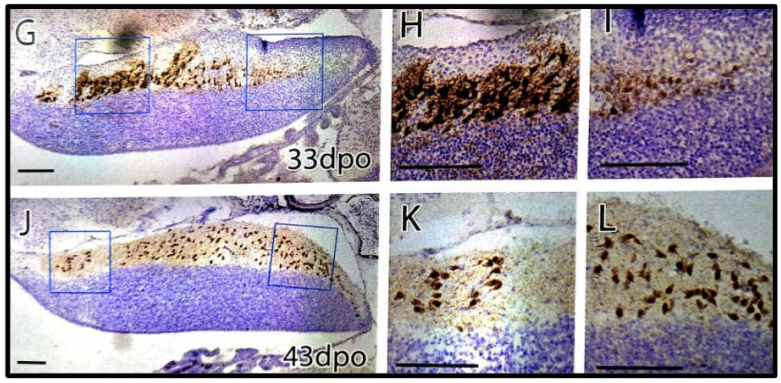


Figure 4: Immunohistochemistry staining for Calbindin of snakes cerebellum. We see Purkinje cells (black-brown cells popping out) scattered all around the Molecular layer. We don't see any Purkinje layer. From Tristan Varela Thesis.[6]

Can we come to the same conclusion and confirm Tristan's finding? Do other species of snakes show the same cerebellum structure? Why are the Purkinje cells spread around the molecular layer? What impact does it have on snakes welfare and development? Is Reelin responsible for this?

To answer some of these questions, I looked at the positioning of Purkinje cells in snakes brains. To do so, I performed three kinds of experiences: Nissl stains, Immunohistochemistry stains, and Golgi stains. The goal of my research project was to identify the 3-dimensional ordering of Purkinje cells flattened dendritic trees to see if their alignment allows the passage of parallel fibers.

How did I end up doing this internship?

I am interested in Evolutionary Biology, Developmental biology, Ecology, Genetics, Zoology, and Ethology. I am also keen on ornithology, herpetology, aquariophilie and entomology. At a younger age, I used to bread *Pantherophis guttatus* snakes. After making an internship on "Comparative anatomy and squamates morphology" with Aurélien Miralles at the Muséum National d'Histoire Naturel, I met Dr. Wingate who was working on cerebellum development in reptiles. By talking to him a little, I became really enthusiastic about his research and postulated rapidly to an internship with him next summer. My previous knowledge of herpetology and interest in developmental biology helped me to get through this internship successfully.

Material and Methods

To answer my research question arising from Purkinje cells in snakes cerebellum during this internship, I needed to use three different methods: the Nissl Staining, Immunohistochemistry staining, and the Golgi staining. Nissl staining was to see the structure of cells stratification of the snake's cerebellum. I wanted to verify that the cerebellum looked like scientists though it is supposed to be. The immunohistochemistry staining was to see how ordered were the Purkinje cells in 2 dimensions, how they were aligned with one another. The Golgi staining was to look at their actual morphology and 3d structure. It is the Golgi staining that was supposed to give a conclusive answer, the other stainings only assure what we already know. The brain of two species of snakes at different development stages was used, *Pantherophis guttatus* and *Psammophis sibilans*.

PARAFFIN SECTIONING METHOD FOR BRAIN TISSUES

The protocol below is used for both the Nissl stain and the Immunohistochemistry stain. It was taken from "Paraffin Processing of Tissue." Protocols Online, June 24, 2010. https://www.protocolsonline.com/histology/sample-preparation/paraffin-processing-of-tissue/. [7]

Description: This method is used to get thin paraffin sections from 5 - 30 µm of brain tissues which will be used next for staining. In the tissue preparation, the tissue is dehydrated and then infiltrated with wax before being embedded into wax blocks. Once the tissue is embedded, it can be stored in the cassettes at room temperature indefinitely.

Detailed Protocols: **See ANNEX 1 :** [7]

- **Tissue preparation:**

Thickness: No more than 3 mm thick tissues.

Area: 20 mm × 30 mm.

After collecting your samples, this part prepares the tissue before embedment. This step takes approximately 60 hours. Clean the tissues by washing your brains 2 times with distilled water, then put them through different bathing of alcohol. Then you put them in hot liquid wax. Tissues are now ready for embedments.

- **Embedding tissues in paraffin blocks:**

After having processed the tissue in the previous step, this part stores it in cassettes. This step takes approximately 1 hour, don't forget to pre-melt the paraffin which adds 1 hour overall. Embed the tissue by putting them in a metallic cassette filled with hot wax. Remove the metallic cassette after orientating the brain and letting the wax cool down and solidified. Your tissues are ready for cutting.

- **Sectioning tissues using a microtome:**

Now that you have your tissue block, this step section the tissue before staining. This part takes a completely different amount of time depending on the number of slices you are making. Section the tissues using a microtome machine. Tissues are now ready for microscopic observation and staining.

Result: We obtain slides with thin paraffin section of snakes brain.

NISSL STAINING METHOD AND PROTOCOL ON PARAFFIN SECTIONS FOR BRAIN TISSUES

Protocols of Nissl staining were taken from Accessed December 1, 2019. https://biomedical-sciences.uq.edu.au/files/57/Nissl%20Staining%20Method%20brain.docx. [8]

Description: This method is used for the detection of neurons. The cresyl violet used in the protocol stains the negatively charged RNA in blue-violet. Nissl bodies present in the cytoplasm of neurons contain RNA, thus, they are dye in blue-violet. This technique is used on paraffin-embedded tissue sections, fixated with paraformaldehyde. This staining was done to identify the basic neuronal structure and cell stratification of snakes cerebellums.

Fixation: 4% paraformaldehyde in 0.1M PBS.

Section: paraffin sections from 5 - 30 µm.

Protocol of tissue staining:

Use the slides you prepare previously, and stain them using cresyl violet. Slices go through a diverse number of baths of alcohol and Xylene, a clearing agent. This part takes approximately 110 minutes. Nissl staining is now done, tissues are now ready for microscopic observation.

Detailed Protocols: **See ANNEX 2** [8]

Results: Neurons, more precisely the Nissl Body, are highlighted in purple-blue.

IMMUNOHISTOCHEMISTRY: IMMUNOSTAINING PARAFFIN WAX SECTIONS

Protocols of Immunohistochemistry were given to me by Carl Hobbs, research engineer at KCL.

Description: This method is used to identify antigens in cells by exploiting the principle of antibodies binding specifically to antigens in biological tissues. This staining was done to detect Purkinje cells. The Calbindin D Antibody used here turns brown. Calbindin D is present in Purkinje cells but not in Granule cells, therefore, Purkinje cells are dyed brown. This technique is used on paraffin-embedded tissue sections. This staining was done to see in 2D how ordered were the Purkinje cells of snakes cerebellums. Purkinje cells are colored in brown, all the other cells are colored in blue.

Section: paraffin sections from 5 - 30 µm.

Immunohistochemistry Protocol: Immunohistochemistry selectively identifies antigens (proteins) in Purkinje cells of the cerebellum slices by exploiting antibody-antigen interaction. Calbindin D Antibody, which binds specifically to antigens in biological tissues, is also conjugated to peroxidase enzyme that catalyzes a color-producing reaction. Multiples antigens are used here to test the one that works best and that will show the best coloring results: Goat anti-rabbit, Goat anti-mouse, Mouse anti-goat, and anti-Calbindin D. The only antigens to show us an appreciative coloring is the anti-Calbindin D. The tissue passes through a antigens solution, then rinse briefly by a buffer. Afterward, it goes on a Calbindin D antibody solution which will trigger the coloring reaction.

Detailed Protocol: **See ANNEX 3**

Results: Antigen sites are colored in brown; Nuclei are colored in blue.

GOLGI STAINING METHOD AND PROTOCOL ON AGAROSE SECTIONS

The protocols of the Golgi staining were inspired by Czechowska N. et al, 2019. None were taken fully from existing papers. I created specific protocols assembling parts of existing techniques to construct the best method to stain Purkinje cells only. [9]

Description: This method is used to visualize nervous tissue under light microscopy. It is a silver staining technique that dyes black a limited number of cells at random in their entirety. Here, we want to study Purkinje cells, so we adapted the already known protocols to one that would best display the cells of interests. Because researchers do not know how this method stains cells, we could not refer to most papers, that were studying different kinds of cells. This technique is used on agarose-embedded tissue sections. This staining was made to lock at the actual morphology and 3D structure of neurons in snakes cerebellums. Two different QGF were prepared, we called them QGF1 and QGF2. The preparation of both QGF was made under a chemical hood.

Section: agarose sections from 200 - 400 µm.

Preparation:

QGF is prepared to allow the coloring of snakes' brains. QGF is made of a mix of fixing agents, oxidizing agents, alcohol, and silver nitrate. Another variant of QGF with reagents supposed to increase the final staining of the brain was made using Triton and sucrose. To stain brains using the Golgi method, bath your tissue in fixating agent, then immerse it in your QGF solution, afterward rinse it with multiple alcohol solutions.

Detailed Protocols: **See ANNEX 4 :** [9]

- QGF1 Preparation Protocol
- QGF2 Preparation Protocol
- Golgi's Impregnation Protocol
- Slice Dissection and Vibratoming in Agarose [10]

Results: Neurons, more precisely the Purkinje cells, are stained black on a light background.

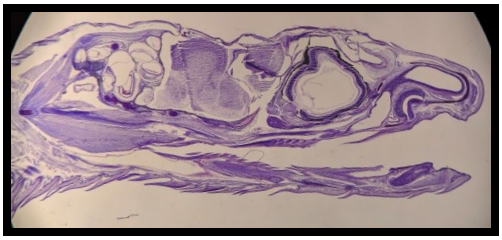
Results and discussion

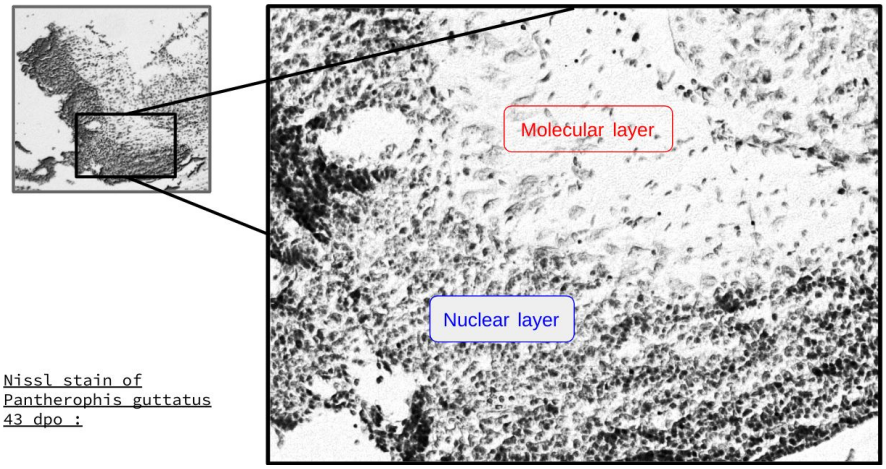
All pictures shown in this part of the report were taken using my phone or a digital microscope camera.

Accordingly to the previous assumption made by Tristan Varela in his thesis, Nissl staining successfully showed that cell stratification of the snake's cerebellum is similar to those of other animals. Indeed, we can clearly identify the molecular and nuclear layers (Figure 6) in the cerebellum. However, not all the slice shows the cerebellum (Figure 5). We explicitly distinguish Purkinje cells in the molecular layer (Figure 6 and 7). Moreover, the results of the Nissl stain goes in the same way as Tristan's assumption. As such, we slightly see that Purkinje cells are not all well aligned in an arranged layer, the Purkinje layer (Figure 7). They are more scattered than on other animals. So, this experience reinforced the assumption that snakes do not have a Purkinje layer. With the supplementary results I brought during my internship affirming Tristan's thesis, Richard Wingate thinks there is now enough data to publish a paper.

Even if we do notice Purkinje cells migrating from the ventricular zone toward the outer surface of the cerebellar cortex during snakes embryogenesis when the cerebellum develops, the results were not as conclusive (Figure 8). Indeed, even if we guess Purkinje cells migration, we do not easily distinguish them from granule cells. This is due to the number of cells compacted in the cerebellum during the first stages of cerebellar development.

Figure 5: Nissl stain of Pantherophis guttatus 43 DPO (days post-ovulation) on the binocular loupe. Full head staining. Here the cerebellum can not be seen.





Nissl stain of Pantherophis guttatus 43 dpo :

Figure 6: Nissl stain of Pantherophis guttatus 43 DPO (days post-ovulation) cerebellum on the microscope in black and white with a legend. We can easily identify the Purkinje cells in the Molecular layer (ovoid grey cells) and Granule cells in the Nuclear layer (smaller black cells look like dots)

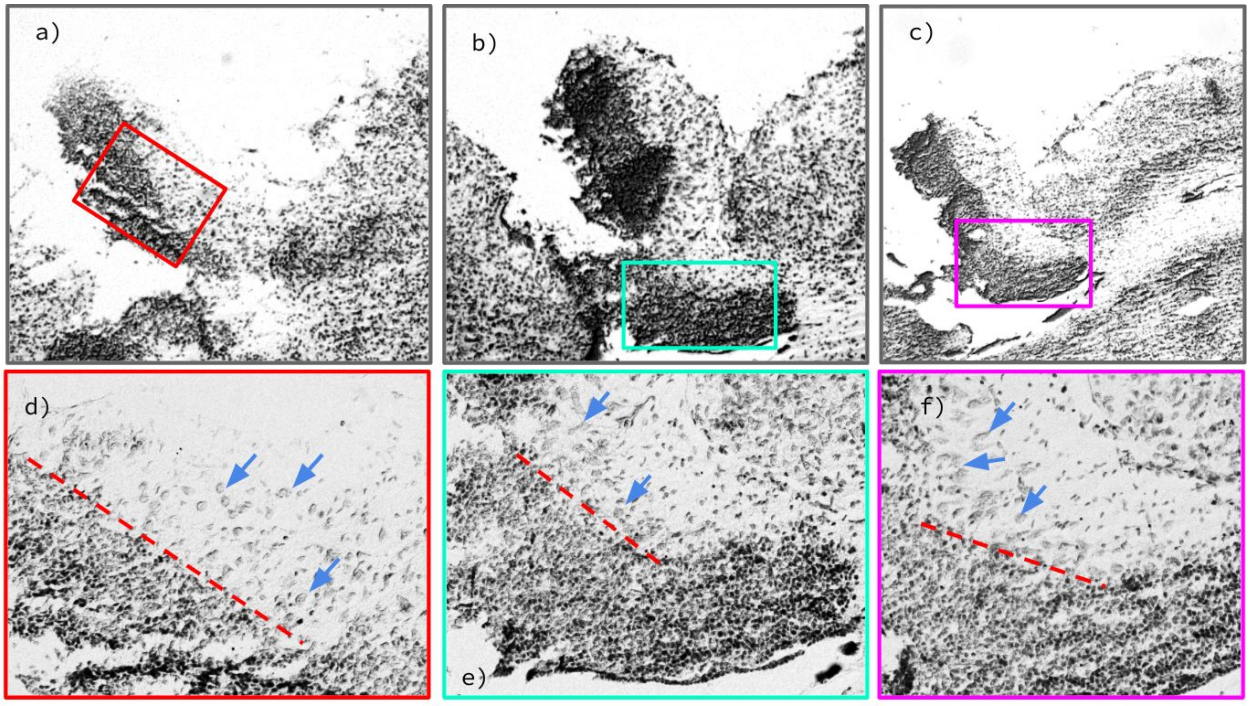


Figure 7: Nissl staining of Pantherophis guttatus 43 DPO (days post-ovulation) showing the cerebellum on microscope in black and white. a, b, c views are x40 ; d, e, f views are x200. d, e, f views are a zoom of respectively a, b, c views. We can clearly distinguish the molecular and nuclear layers (molecular layer on the upper right of the red dashed line, nuclear layer on the other side of the red dashed line). We also notice Purkinje cells (blue arrows ⇒ not all Purkinje cells are pointed by the arrows) that are not ordered in a thin line but spread through the molecular layer. There is no Purkinje cell layer.

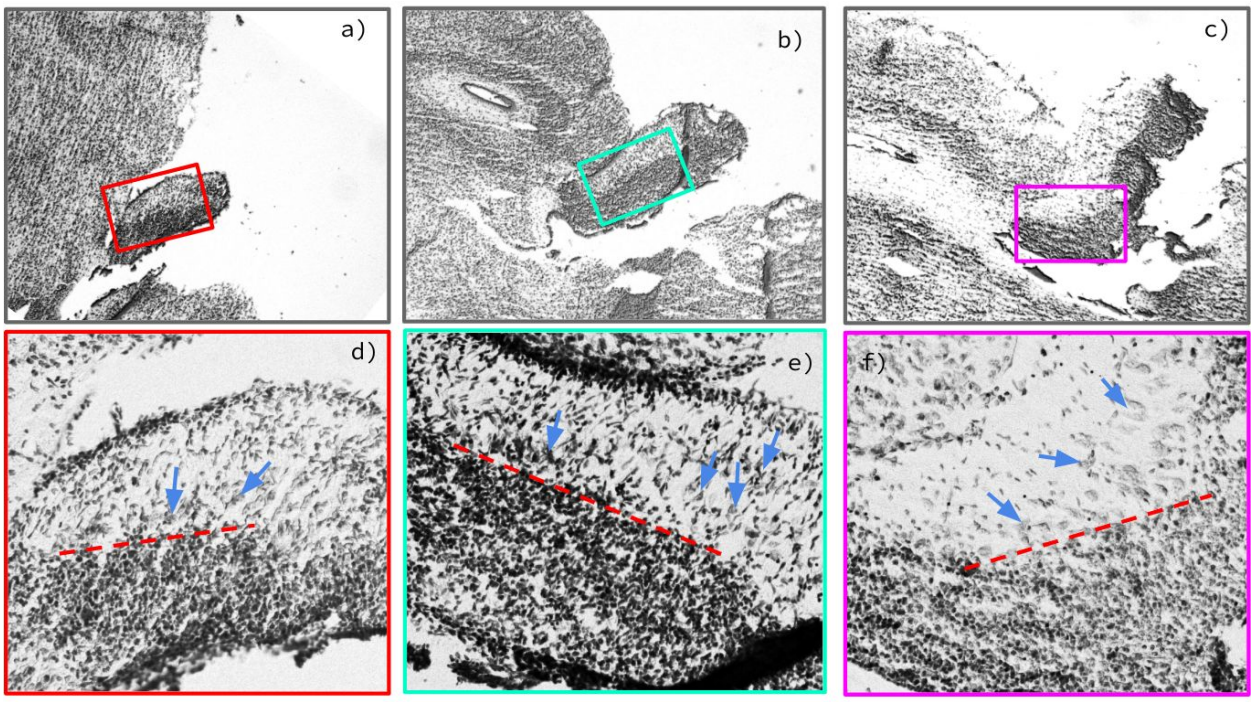


Figure 8: Nissl staining of Pantherophis guttatus at 3 stages of development : 29 DPO (a and d views), 33 DPO (b and e views), and 43 (c and f views) DPO (days post-ovulation), showing the cerebellum development on microscope in black and white. a, b, c views are x40 ; d, e, f views are x200. d, e, f views are a zoom of respectively a, b, c views. We can clearly distinguish the molecular and nuclear layers (molecular layer above the red dashed line, nuclear layer below the red dashed line). We also notice Purkinje cells (blue arrows ⇒ not all Purkinje cells are pointed by the arrows) migrating from the ventricular zone in the neural tube where they arise, toward the outer surface of the cerebellar cortex.

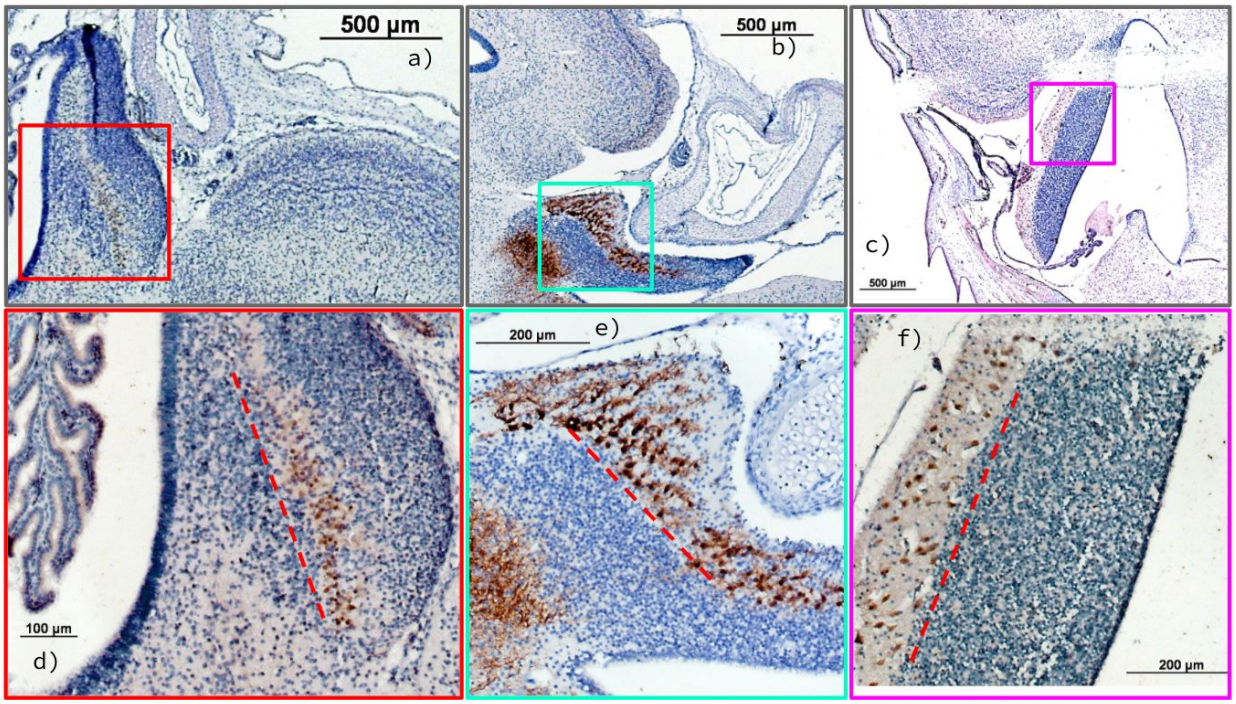


Figure 9: Immunohistochemistry staining for Calbindin of Psamophis sibilans at 3 stages of development : 25 DPO (a and d views), 33 DPO (b and e views), and 43 (c and f views) DPO (days post-ovulation), showing the cerebellum development on microscope. a, b, c views are x50 ; d, e, f views are x200. d, e, f views are a zoom of respectively a, b, c views. We can clearly distinguish the molecular and nuclear layers on c and f views (molecular layer on the side of the dashed red line with the brown dye, nuclear layer on the other side). We distinguish clearly Purkinje cells in a brown coloration migrating through the cerebellum during its development. We can see on views e and f the apical dendrites extending from the nucleus. This gives us the direction of alignment of Purkinje cell dendrites, that are supposed to be stacked one in front of the other. Here, we see that this is not the case. The dendrites seem to be pointing in very different directions, even in opposite ways in some cases. Moreover, nuclei of Purkinje cells are not ordered in a Purkinje layer, rather they are scattered in the molecular layer (views e and f).

In the same way as with the Nissl staining, Immunohistochemistry staining allow us to identify the molecular and nuclear layers (Figure 9). Purkinje cells present in the molecular layer pop in brown, granule cells are in a violet-blue (Figure 9 and 10). Immunohistochemistry staining gives

us information about the stacking of Purkinje cells, and their large dendritic arbors that form nearly two-dimensional layers through which parallel fibers from the deeper-layers pass. Here, we can see the apical dendrites extending from the nucleus of the Purkinje cells. Apical dendrites give us a rough idea of the global direction of the dendritic arbor. In some cases here, by approximating dendrites position, they point in opposite directions (Figure 10). Thus, we can state that none of the less, Purkinje cells nuclei are not ordered to form the Purkinje layer, but moreover, the two-dimension side of dendritic arbors are not stacked together like pages of a book. This means that dendritic arbors can not all be interconnected by parallel fibers from the deeper-layers. Therefore, they do not communicate with the rest of the body.

With the Immunohistochemistry staining, we can look through the migration of Purkinje cells from the ventricular zone toward the outer surface of the cerebellar cortex during snakes embryogenesis when the cerebellum develops (Figure 9).

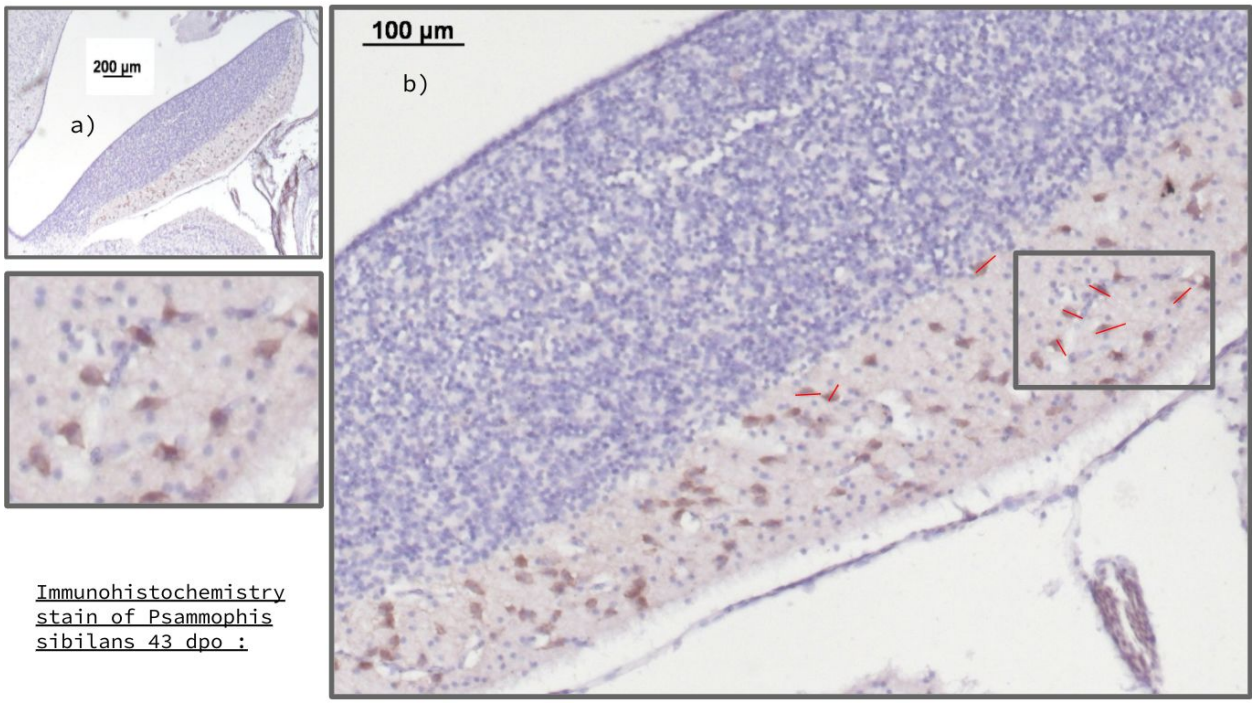


Figure 10: Immunohistochemistry staining for Calbindin of Psammophis sibilans at 43 DPO, showing the cerebellum development on microscope. a view is x50 ; b view is x200. b view is a zoom of a view, and the last picture is a zoom of the rectangle on b view. We distinguish Purkinje cells in brown coloration. We can see on views b the approximate orientation of apical dendrites extending from the nucleus shown by a red line. This gives us the direction of alignment of Purkinje cell dendrites, we observe that they are pointing in different directions. We concentrated our observation on a small part of the cerebellum to see the orientation of dendrites (the rectangle on b view).

Golgi staining revealed the actual morphology and 3d structure of Purkinje cells, which was yet unknown. Only Purkinje cells were colored by the Golgi staining, other kinds of cells are not visible (or only in a few numbers). So the QGF solutions supposed to enhanced Purkinje cell coloring successfully worked. It helps us identify the molecular and nuclear layers. We can identify the apical dendrites extending from the nucleus of the Purkinje cells (Figure 12). We observe that dendrites are globally oriented in the same direction, even if cells' bodies are not stacked in an orderly line. The Golgi staining also gives us a perspective of depth (Figure 11). A cell dendrite can point in different directions depending on the layer you are looking at. It is not clear in which direction are oriented the dendrites, they seem to be pointing in different 3-dimensional direction and orientation.

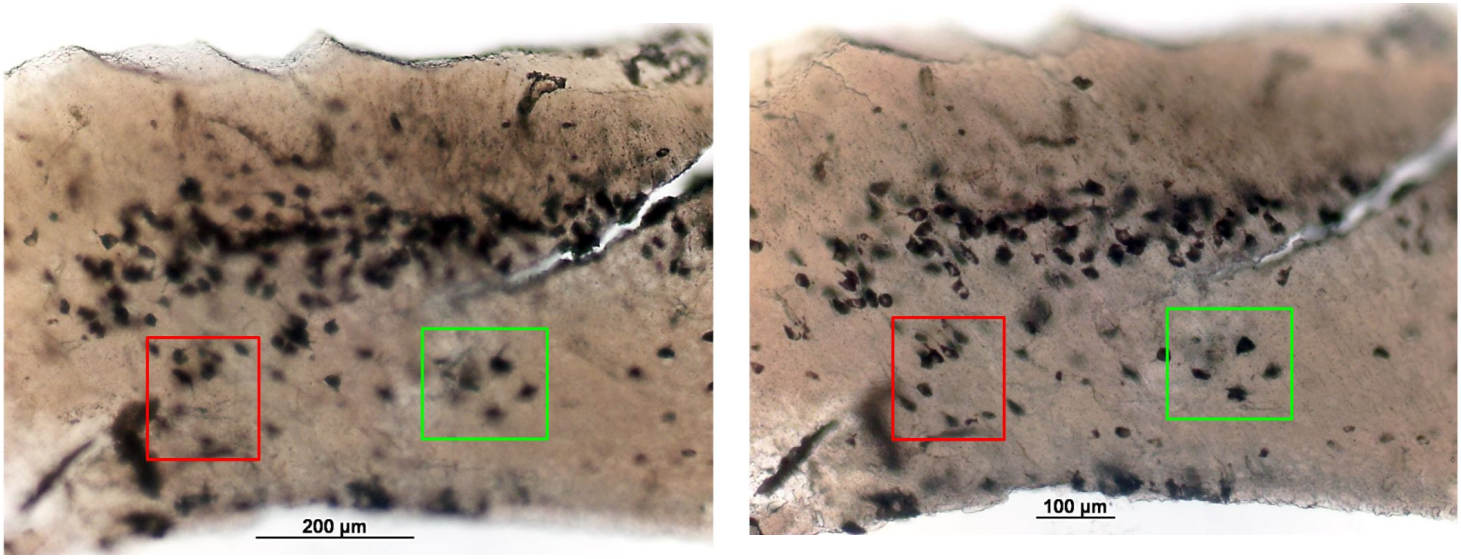


Figure 11: Golgi staining of *Pantherophis guttatus* at 43 DPO, showing the cerebellum under a microscope x200. Both pictures show the exact same area at different optic focus. I decided to focus on two parts of this view that I distinguished by a red and green rectangle. We can distinguish the molecular from the nuclear layers. Only Purkinje cells are visible, so the area densely colored by black cells is the molecular layer, the nuclear layer is on the side poorly populated by cells. Both red and green rectangles show dendrites of the same cell pointing in different directions depending on the focus. I interpret that dendrite may be pointing upward compared to the picture. However, not all cells on these pictures have dendrites facing upward, so they may be orientated in different 3-dimensional directions.

Something important to notice is that neither the immunohistochemistry nor the Golgi staining shows the actual dendritic arbor. Usually, with both of those staining, a part of apical dendrites can be seen. So why can't we see Purkinje cell dendritic foliation? Maybe Purkinje cells in snake cerebellum does not have flattened dendritic trees, or maybe stainings don't display it.

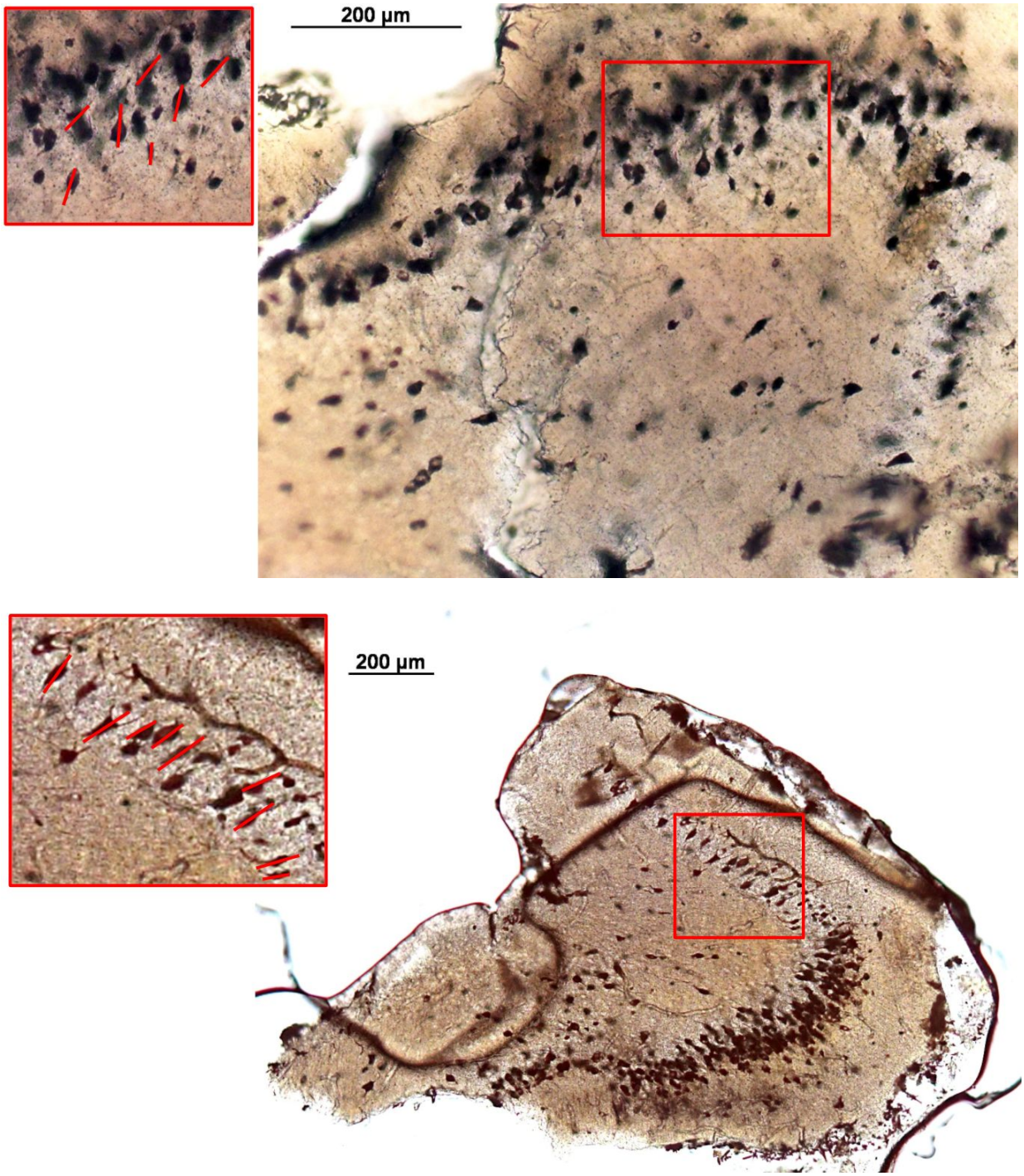


Figure 12: Golgi staining of *Pantherophis guttatus* at 43 DPO, showing the cerebellum under a microscope x200 (first picture) and x100 (second picture). I focused my attention on one part of each picture represented by a red rectangle. I represented the general direction of the apical dendrites extending from the nucleus by a red line. We can recognize the molecular layer (the area densely populated by cells, knowing that only Purkinje cells are visible by Golgi stain). We can not identify any Purkinje layer. In both pictures, the dendrites seem to be pointing in similar directions (the red lines are oriented in the same way)

Conclusion and perspectives

Cell stratification of the snake's cerebellum is similar to those of other animals. They have a molecular layer composed of Purkinje cells and a nuclear layer composed of Granule cells. However, Purkinje cells are oddly position in snakes' cerebellum. They are not ordered in a Purkinje layer like in all the other vertebrates, rather, they are scattered through the molecular layer. Moreover, Purkinje cells' dendritic arbors are not stacked together like pages of a book, they can even point in different 3-dimensional direction and orientation depending on the depth. So they can not all be interconnected by parallel fibers from the deeper-layers. Therefore, they may not communicate with the rest of the body.

We were also able to observe and confirm Purkinje cells migration from the ventricular zone toward the outer surface of the cerebellar cortex during snakes embryogenesis when the cerebellum develops.

I successfully made a Golgi staining, that was coloring the exact cells we wanted to see, the Purkinje cells. It was the first Golgi staining made in this department. During this internship, I refined and made a Golgi staining technique of Purkinje cells that can be used later on further notice.

I came to the same conclusions as Tristans' and confirmed his findings. Other species of snakes show the same characteristics of Purkinje cells alignments. I gathered enough data for a possible future scientific publication on snakes' peculiar cerebellum.

To go further, we could look for the expression of Reelin protein in snakes and see if there is a correlation with their Purkinje cells alignments. We could study the cerebellar structure of other limbless vertebrates (vertebrate that does not have limbs but has an ancestor that did like slowworm, or Amphisbaenia, or caecilians, or Sirenidae) and vertebrates with extremely atrophied limbs that appear non-functional (like Amphiumidae). This study could show a correlation between limbs and Purkinje cells repartition in vertebrates. Dr. Wingate and I talked about the feasibility and outcomes of interest in studying the cerebellar structure of other limbless vertebrates. It might lead to another internship next summer.

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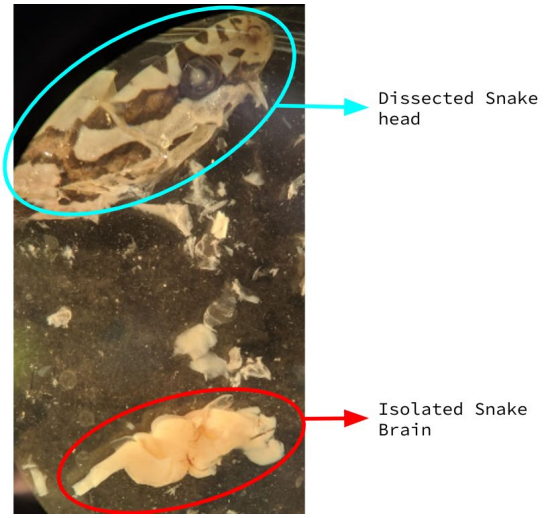
Snake picture 1: https://www.pestwiki.com/corn-snake/

Snake picture 2: https://undergroundreptiles.com/shop/hissing-sand-snake-2/

Supplementary material

ANNEX 1: PARAFFIN SECTIONING METHOD FOR BRAIN TISSUES

Figure 13: Step 1 ⇔ extraction of the brain from the head of the snake for staining purposes



1) Extract the brain from the head of the snake if you want a section with no other tissue *OR* keep the all head.
2) Store in neutral buffered formalin for 48 hours to fixed tissue.
3) Trim fixed tissues and keep in neutral buffered formalin until ready to proceed. Put tissues in a labeled (usually with pencil, as solvents dissolve the ink) cassette.
4) While shaking gently, put the tissue in 70% ethanol for 1 hour.
5) While shaking gently, put the tissue in 95% ethanol (95% ethanol / 5% methanol) for 1 hour.
6) While shaking gently, put the tissue in the first absolute ethanol for 1 hour.
7) While shaking gently, put the tissue in the second absolute ethanol for 1½ hours.
8) While shaking gently, put the tissue in the third absolute ethanol for 1½ hours.
9) While shaking gently, put the tissue in the fourth absolute ethanol for 2 hours.
10) While shaking gently, put the tissue in the first clearing agent Xylene for 1 hour.
11) While shaking gently, put the tissue in the second clearing agent Xylene for 1 hour.
12) While shaking gently, put the tissue in the first wax at 58°C for 1 hour.
13) While shaking gently, put the tissue in the second wax at 58°C for 1 hour.

The tissue is now processed, it can be stored in the cassettes at room temperature indefinitely.

Embedding tissues in paraffin blocks:

1) Turn the heat block on to melt the paraffin 1 hour before adding the tissue cassettes.

2) Melt the wax placing the entire cassette in 58°C paraffin bath for 15 minutes. (wax melting temperature is 60°C)
3) Open cassette to view the tissue sample and choose a mold that best corresponds to the size of the tissue.
4) Discard cassette lid.
5) Put a small amount of molten paraffin in mold, dispensing from the paraffin reservoir.
6) Using warm forceps, transfer tissue into the mold, placing cut side down, as it was placed in the cassette.
7) Transfer mold to a cold plate, and gently press the tissue flat. Paraffin will solidify in a thin layer which holds the tissue in position.
8) Add the labeled tissue cassette on top of the mold as a backing.
9) Wait approximately 30 mins for the wax to be completely cooled and hardened, then popped out of the mold the paraffin block.

The tissue and paraffin attached to the cassette have formed a block, which is ready for sectioning. Tissue blocks can be stored at room temperature for years.

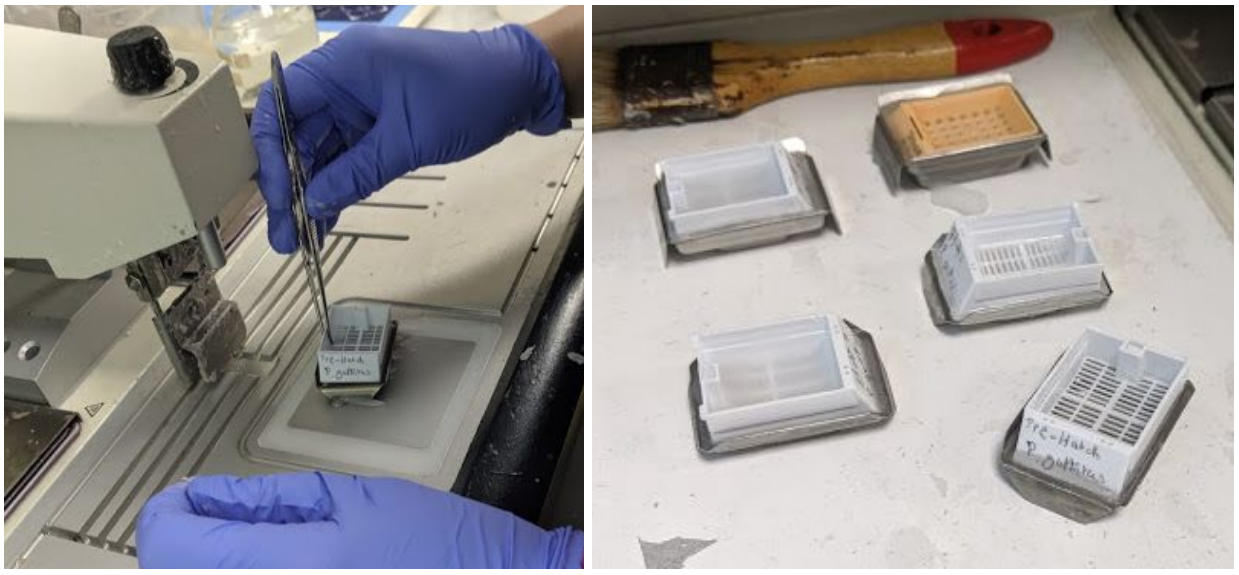


Figure 14: Representation of the steps 7 to 10. The mold is on a cold plate to allow solidification of the paraffin before popping out of the mold the paraffin block (on the left / Victoria Rook hands). Labeled cassettes in the mold hardening for 30 min (on the right).

Sectioning tissues using a microtome:

1) Turn on the water bath and check that the temp is 35-55ºC. Fill with fresh deionized water.
2) Place the blocks you want to sectioned face down on an ice block.
3) Place a fresh blade on the microtome and angle it between 5 and 10º. Insert the block into the microtome chuck so the wax block faces the blade and is aligned in the vertical plane.
4) Set the dial to cut 60 µM sections in order to plane the block; once it is cutting smoothly, set to the paraffin section size your interested in, here between 5 to 30 µM.
5) Cut down the block to obtain sections and pick them up with forceps and a fine paintbrush.
6) Float the cut sections on the surface of the water bath.

If the specimens fragment when placed on the water bath then it may be too hot.

7) Float the sections onto the surface of clean glass slides.
8) Place the slides with paraffin sections on the warming block in a 65°C oven for 20 minutes (so the wax just starts to melt) to bond the tissue to the glass.

Slides can be stored overnight at room temperature. It is better to conserve them at 4°C.

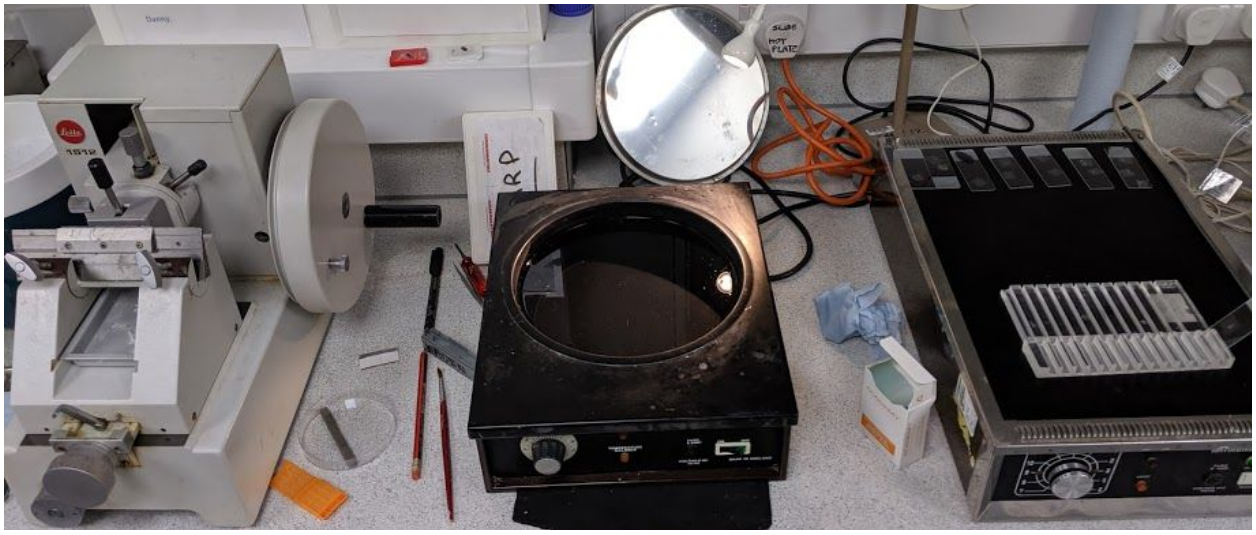


Figure 15: On the left the microtome, on the middle the water bath, on the right the warming block I used

Result: We obtain slides with thin paraffin section of snakes brain.

ANNEX 2: NISSL STAINING METHOD AND PROTOCOL ON PARAFFIN SECTIONS FOR BRAIN TISSUES

For the protocols below, when no information is given about the quantity of liquid used to bath the brain tissues, put a decent amount to immerse them completely.

Protocol of 0.1% Cresyl violet solution:

1) Add 0.1 g of Cresyl violet acetate.
2) Add 100 mL of dH_2O (distilled water).
3) Add 10 drops of glacial acetic acid just before use and filter.

Protocol of tissue staining:

1) Dewax 10 mins in Xylene.
2) Dewax 10 mins in Xylene.
3) Dewax 10 mins in Xylene.
4) Wash 5 mins in Absolute Alcohol.
5) Wash 5 mins in Absolute Alcohol.
6) Wash 3 mins in 90% Alcohol.
7) Wash 3 mins in 70% Alcohol.
8) Wash 2 mins in Running Water.
9) Rinse in tap water and then in ddH_2O (double distilled water).
10) Stain in 0.1% cresyl violet solution for 3-10 minutes.
11) Rinse quickly in ddH_2O (double distilled water).
12) Differentiate in 95% ethyl alcohol for 20 minutes.
13) Wash 2 mins in Absolute Alcohol.
14) Wash 2 mins in Absolute Alcohol.
15) Wash 2 mins in Absolute Alcohol.
16) Clear 5 mins in Xylene.
17) Clear 5 mins in Xylene.
18) Clear 5 mins in Xylene.
19) Mount slides with coverslips using DePeX

Results: Neurons, more precisely the Nissl Body, are highlighted in purple-blue.

ANNEX 3: IMMUNOHISTOCHEMISTRY: IMMUNOSTAINING PARAFFIN WAX SECTIONS

1) Buffer solution

I -TBS "Immunobuffer wash"

Protocol of the TBS Immunobuffer wash at 10X TBS Stock Solution (pH7.6, 0.5M):

1) Take 30 g of TRIS.
2) Add 40 g NaCl.
3) Dissolve in 350 ml deionized water. Adjust to pH7.6 using 25% HCl.
4) Top up to 500ml.

Protocol of the TBS Immunobuffer wash at 1X TBS - Working solution:

1) Take 50ml TBS stock solution (pH7.6)
2) Add 450 ml deionized water.

II -TB "DAB Developing buffer"

Protocol of the TB DAB Developing buffer at 10X TRIS Buffer (pH7.6 1M):

1) Take 60 g TRIS.
2) Add 350 ml of deionized water. Adjust to pH 7.6 using 25% HCl.
3) Top up to 500 mL.

For x1 working solution, see DAB solution SOP.

2) Blocking buffer and primary/secondary antibody diluent

Protocol of the 2% BSA in 1x TBS and Azide, pH7.6:

1) Take 4g of BSA (Sigma A4503)
2) Add 20ml 0.5M TBS (pH7.6)
3) Add 180ml deionized water.
4) Add 2ml of 10% Sodium Azide (NaAz).
5) Dissolve, then store at 4 degrees Celsius.

3) Antigen retrieval solution

I - Citric Acid Solution

Citric Acid Stock Solution

1) Take 105g of Citric Acid.
2) Makeup to 500ml using deionized water.

Citric Acid Working Solution

1)
    - For I rack: 8ml stock solution + 800ml deionized water.
    - For 2 racks: 13ml stock solution + 1300ml deionized water.
2) Adjust to pH6 using 5M NaOH.

II - Protease digestion: see individual SOPs

Immunostaining solutions

Goat anti-rabbit	1 : 300	Vectoriab BA-1000
Goat anti-mouse	1 : 300	Vectoriab BA-9200
Mouse anti-goat	N/A	N/A
Anti-Calbindin D	N/A	N/A

Procedure:

1) Cut paraffin sections mount on “superfrost plus”.
2) Drain for at least 60 mins or overnight in a fume hood.
3) Leave for 30 mins in the 60 degrees Celius oven immediately before dewaxing (warm slides dewax in a shorter time)
4) **Dewax:**
    - Bath in xylene, 2 times, 5mins with agitation.
    - Bath in 100% IMS, 4 times, 2mins with agitation.
5) **Incubate:** for 10mins in 3% hydrogen peroxide.
6) Prepare Antigen retrieval solution: Microwave (1000W) — Pressure cooker
    a) Place Antigen retrieval working solution into pressure cooker base, adjust pH and heat on full power until start of boiling (~ 11 mins in a 900W microwave)
    b) Place the rack of slides into solution, laying it on its side, put the lid and pressure valve on, and heat in microwave on full power for 5 mins.
    c) When it has finished, open the microwave door and leave to cool until the yellow valve is fully depressed (approx 15 mins).

d) Take the cooker to the sink, take the lid off and allow cold tap water to gently run onto the rack, cooling the slides and removing all traces of buffer (approx 10 mins). CARE! Boiling solution

Antigen retrieval: 90C oven.

7) **Wash:** rinse thoroughly in slow running tap water.

Immunostaining Procedure:

1) Drain slides briefly by standing on absorbent tissue.
2) Ring each section using a 'PAP' pen.
3) Cover sections with blocking solution and incubate for at least 5 minutes while making up the primary antibodies.
4) Rinse slides in 1x TBS.
5) Wash for 10mins in 500ml container of 1x TBS, with stirring.
6) Flick off excess TBS, Incubate for 60mins at WT in biotinylated secondary antibody, diluted in blocking buffer
7) Immediately make up the StreptABC-HRP, and leave to conjugate for 30 minutes.
8) Rinse slides in 1x TBS.
9) Wash once for 5mins in the same 500ml 1x TBS, with stirring.
10) Incubate in StreptABC-HRP for 30mins at room temperature.
11) Rinse off with 1x TBS and wash for 5mins in the same 500ml TBS, with stirring.
12) Develop for 10 mins in DAB solution, gently agitate.
13) Wash under running tap water for 5mins.
14) Counterstain using Haemalum -2mins
15) Wash under a running tap until clear.
16) Differentiate using 0.5%HCl in 70% IMS.
17) Wash under running water. Check the optimal intensity of nuclei under a microscope.
18) **Dehydrate:** rinse thoroughly in 100% IMS, 4 times 2mins.
19) **Clear:** rinse thoroughly in xylene, 2 times 5mins.
20) **Or:** after Haemalum has blued, rinse slides in dist water, place in 60 degrees Celsius oven to dry. After 60 mins, rinse in 100% alcohol, then continue with step 19.
21) **Mount:** using a solvent-based plastic mountant, here DPX. Leave to set.

Results: Antigen sites are colored in brown; Nuclei are colored in blue.

ANNEX 4: GOLGI STAINING METHOD AND PROTOCOL ON AGAROSE SECTIONS

QGF1 Preparation Protocol (for 100 mL):

1) Add 5 g of potassium dichromate.
2) Add 5 g of chloral hydrate.
3) Add 4.4 g of 4.4% formaldehyde.
4) Add 10 drops of DMSO (Dimethyl sulfoxide).
5) Add 8 mL of 2% glutaldehyde.
6) Complete to 100 mL with ddH_2O (double-distilled water).
7) You obtain an earthy-reddish solution. Verify that the solution is at pH 6. If not, use NaOH or HCl to get the QGF to pH 6.

QGF2 Preparation Protocol (for 100 mL):

1) Add 5 g of potassium dichromate.
2) Add 5 g of chloral hydrate.
3) Add 4.4 g of 4.4% formaldehyde.
4) Add 15 g of 15% sucrose.
5) Add 10 drops of DMSO (Dimethyl sulfoxide).
6) Add 8 mL of 2% glutaldehyde.
7) Add 100 mL of 0.1% Tritton X.
8) Complete to 100 mL with ddH_2O (double-distilled water).
9) You obtain an earthy-reddish solution. Verify that the solution is at pH 6. If not, use NaOH or HCl to get the QGF to pH 6.

Golgi's Impregnation Protocol :

1) Cut the brains into half along the middle line.
2) Fix the hemispheres in 20 mL of 4% paraformaldehyde for 48h.
3) The tissue is chromated in QGF in the dark for 24h.
4) Increase the QGF's pH from 4.3 to 5.3 to 6.3 with 8h intervals while the tissue rest in the dark for 24h more hours (3*8 hours).
5) Rinse briefly the tissue in 0.75% silver nitrate :
    a) Take a cup of silver nitrate 0.75%;

b) Transfer the brains from the QGF to the silver nitrate;
c) Put the cup into aluminum foil (darkness) and shake it gently for 5 min,
d) Transfer the brains into a new cup of silver nitrate, put the cup into aluminum foil (darkness) and shake for 5 more min;
e) Continue the process until the silver nitrate stays clear from the dark coloration of the brains.

6) Impregnate the tissue in 20 mL of 0.75% silver nitrate while shaking in the dark for 48h.
7) Wash tissue in 70% ethanol, leave for 1 hour :
    a) Remove the silver nitrate;
    b) Put a fair amount of alcohol;
    c) Leave it for 1 hour shaking gently in the dark;
    d) Put a new batch of alcohol and repeat another time.
8) Embed the brain tissues in agarose gel and let it dry :
    a) Preparation of 2% agarose gel :
        (1) Weigh out 2 g low melting temp agarose;
        (2) Put it into a glass jar and add 100 mL of TAE;
        (3) Shake briefly;
        (4) Leave in the oven at 60 degrees overnight;
    b) Embed the Golgi's stained brains in agarose gel :
        i) Put the brains into small cassettes;
        ii) Add the liquid agarose;
        iii) Let the cassettes dry in the fridge at 4 degrees to accelerate the agarose gel solidification procedure.
9) Cut fin slice (50 - 200 µm thick sections) :
    a) Remove the cassettes from the fridge;
    b) Disasosiate the cassettes from the dry agarose gel;
    c) Glue the agarose gel to the specimen holder;
    d) Using a Vibratome sectioning machine, cut fin slice;
    e) Mount the slice on a microscope slide;
    f) Remove carefully the extra PBS (vibratome slicing is done in cold PBS) with a tissue;
    g) Add DPX, a solvent base plastic mountant, and the cover glass;
    h) Name each slide;

i) Leave to set.

10) Image the tissue.

Results: Neurons, more precisely the Purkinje cells, are stained black on a light background.

Outcomes

During this internship, I learned to follow precise protocols of staining techniques. I was immersed in a lab with my own responsibility given, and I experienced workflow and professional dynamic of a laboratory. I have been through all the steps of completing a research project, from discovering the field by looking at the accessible literature, to writing a report showing the results obtained. I learned to communicate with others, making connections useful for my future in the scientific community. I learned to work independently with little guidance. Apart from the knowledge this experience brought me, I experienced living by myself, and taking care of myself in another city away from my home.