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OLIVOCEREBELLAR CONNECTIONS IN THE ATLANTIC STINGRAY

by

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Dedication

For my family and friends.

Acknowledgements

I would like to give a world of thanks to everyone who has supported me throughout this endeavor. Their unyielding encouragement has been a tremendous inspiration and driving force during this academic pursuit, as well as many others. I would also like to sincerely thank my various academic and professional mentors, including my faculty at UHCL. This adventure has been a rewarding journey, and I look forward to the next one!

ABSTRACT

OLIVOCEREBELLAR CONNECTIONS IN THE ATLANTIC STINGRAY

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The morphology of the cerebellum in cartilaginous fishes varies from a smooth, relatively simple bi-lobed structure, to an undulated and complex multi-lobed configuration. Little is known about the anatomical connections and functions of the cerebellum in species with a complex cerebellar morphology, such as the Atlantic stingray, which possesses a tri-lobed cerebellum. The forces that drove this elaboration of the cerebellum in stingrays remain unclear. The inferior olive is a major neural center that is found in all vertebrates. Studies in mammals and other vertebrates indicate that the inferior olive has strong neural connections with, and functional influence on, the cerebellum. Therefore, the elaboration of the cerebellar structure in the Atlantic stingray may have been paralleled by an elaboration of the inferior olive inputs to the cerebellum in this species. In the present study the organization of the neural connections between the inferior olive and the three different lobes of the cerebellum in the Atlantic stingray were revealed using neuroanatomical tracing techniques. Compared to studies of the olivo-cerebellar connections in elasmobranch species with a bi-lobed cerebellar structure, there appears to have been an increase in complexity of these connections in the Atlantic stingray. The results of this research contribute to our understanding of the evolution of the brain in cartilaginous fishes, as well as other vertebrates.

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CHAPTER I:

INTRODUCTION

The cerebellum of the Atlantic stingray displays a unique complexity relative to other cartilaginous fishes[9,24-26]. While the cerebellum of primitive cartilaginous fish is a simple and smooth, bi-lobed structure with a primary fissure dividing anterior and posterior lobes, advanced cartilaginous fishes like the stingray have a tri-lobed cerebellum in which the anterior lobe is further split into rostral and caudal lobules (*Figure 1*)[9,24]. Moreover, the cerebellar lobes in the advanced cartilaginous fishes are highly foliated. This specific morphological pattern of cerebellar structure is likely due to functional specializations of the lobes related to behavior in these animals[9,25].

A prior study demonstrated that the stingray cerebellum receives neuronal inputs from many of the same sources as other cartilaginous fishes with a bi-lobed cerebellum, particularly from the diencephalic and brainstem nuclei, including the inferior olive[7,9,24,25]. These inputs were also observed, however, to have a greater segregation in the complex cerebellum. While the accessory optic nuclei and spinal cord were shown to have connections with the anterior rostral lobule and posterior lobe, respectively, the trigeminal and octavolateral nuclei were shown to have connections with the anterior caudal lobule. Furthermore, previously undescribed midbrain neural centers provide massive inputs to the rostral and posterior lobes of the stingray cerebellum[9,24,25]. To date, there has been little research into the neuroanatomical connections of the cerebellum of advanced cartilaginous fishes, and the inferior olive in particular. One study, however, has been performed on a species possessing a relatively simple bi-lobed cerebellar structure the thornback guitarfish, Platyrhinoidis triseriata[7].

Therefore, very little is known about the neuroanatomy and corresponding structural projections of the complex cerebellar structure in Atlantic stingrays. The

proposed study seeks to extend the understanding of the cerebellar structure, implicated functioning, and overall evolution to the cartilaginous fishes possessing a complex cerebellar structure, with a focus on the inferior olive.

The Classification of Cartilaginous Fishes

Of all the vertebrates, fishes constitute the largest class. Upwards of 90% belong to the Osteichthyes or bony fishes, while the remaining members comprise the cartilaginous fish (*Figure 2*)[30]. The latter is distinct from the bony fishes for its cartilaginous endoskeleton, which makes detailing their evolutionary history particularly challenging due to the scarcity of representatives in the fossil records[30]. Extant cartilaginous fishes, however, belong to the Chondrichtyes class and are divided into either holocephalans (chimaeroids) or elasmobranchs. The latter is comprised of the sharks, skates, and rays, with nearly 1,000 living species dating back almost 400 million years (*Figure 3*)[30]. Due to relatively greater fossil evidence, the elasmobranch group represents a significant component of the early evolutionary history of jawed vertebrates[30]. The primitive qualification of their ancestors, termed gnathostomes, was initially characterized anatomically, describing a limited bony skeleton with mandibular and hyoid arches homologous to gill arches observed by early anatomists. This primitive categorization led to physiological studies designed to identify possible phenotypes shared by the last common ancestor[30].

Elasmobranchs can further be divided into sharks, which includes squalomorphs and galeomorphs, and batomorphs (*Figure 4*). Rays, along with sawfishes, skates, and torpedoes, make up the batomorph group. Although the chondrichthyan cerebrum is large and well-developed, with a brain to bodyweight ratio similar to birds and mammals, the more advanced myliobatiforms possess the highest such ratios of any elasmobranch[25,30]. This larger cerebrum may correlate to a more complex cerebellum, particularly with

regard to the number of neurons in the respective cortices[10]. The myliobatiforms, or stingrays, belong to the Batoidea superorder, which is comprised of four superfamilies, in which the Atlantic stingray, *Dasyatis sabina*, is a member of Dasyatidae or the whiptail stingrays (*Figure 5*). The early development of their forebrain closely resembles that of sharks, but the two groups diverge later in evolution with regard to the extent of their respective ventricular cavities[30]. Their separate evolutionary trajectories appear to have led to a similar outcome for their respective cerebellum, however, as both the galeomorph sharks and the batoids both possess a large structure (*Figure 4*). The squatinomorph sharks, on the other hand, do not present a similarly enlarged cerebellar corpus, despite sharing a common ancestor with the batoids. This distinction suggests that galeoaleomorph sharks and batoids evolved their cerebellum independently.

The Cerebellum of Cartilaginous Fishes

The cerebellum evolved early in the evolution of the jawed vertebrates. It is composed of repeated neuronal motifs that act as an adaptive filter in a vast network of neurons[20]. This filter is thought to have evolved to cancel self-generated sensory noise, such as the animals' own movements and actions[20]. In terms of evolutionary innovation, the cerebellum is believed to have developed as a structure of subsumption architecture. This concept suggests that the cerebellum was superimposed onto existing pathways and circuitry over time, adding computational capacity without compromising fundamental functionality[20].

Other than the telencephalon, the cerebellum of cartilaginous fishes is by far the most variable part of the brain in terms of both shape and size[30]. It is well-defined and robustly developed with a central unpaired corpus cerebelli and the paired auriculae[30]. The former is shaped like an inverted tube, and encompasses a large ventricular cavity as it extends rostrally over the tectum mesencepahli and caudally over the medulla

oblongata. The auriculae, on the other hand, are subdivided into two distinct regions - a rostromedial upper leaf and a caudolateral lower leaf. Both auricles possess upper leaves that are connected by a “lower lip” of neural tissue that extends over the fourth ventricles as a distinguishable band[30]. The marked areae octavolaterales is formed by the caudolateral merging of the auriculae into the dorsal parts of the lateral walls of the rhombencephalon[30].

Historically, the extent of the cerebellum in fishes is difficult to identify because the easily recognizable corpus cerebelli lies adjacent to other regions which are essentially cerebellar-like in neural organization[30]. These regions, the auricles and lateral-line areas, are related to the octavolateral sensory system and were considered to form the octavolateral lobe. This part of the brain has been regarded as the phylogenetic base of the vertebrate cerebellum[5,30]. However, cerebellar-like neural circuits are found in other regions of the vertebrate brain and even in invertebrate nervous systems, and the similarities of internal organization do not necessarily imply homology or a common function[5,21,30].

Although the size of the cerebellum does not necessarily vary with the size of the body of the fish, it has been shown to relate to the overall size of the brain[10]. The size of the brain presumably reflects different levels of sensory analysis and motor performance and relates to the relative complexity of the behavior pattern of a particular species[21,29]. However, these types of correlations are particularly challenging for elasmobranchs as the behavior patterns in these animals is not well described[21].

Internally, the wall of the cerebellar corpus is organized into four distinct layers – innermost granular layer, fiber zone, Purkinje cell layer, and outer molecular layer[13,36,37.] The latter contains several elements – granule cell axons and branches (i.e., parallel fibers), Purkinje cell dendrites, possibly climbing fibers from neurons in the inferior

olive, and stellate interneurons. Whether climbing fibers are present in the cerebellum of fishes has been debated1. The inferior olivary nucleus, the chief source of climbing fibers in the mammalian cerebellum, has long been recognized in many elasmobranch species.[21,22] Several studies in cartilaginous fishes have shown the neurons in this nucleus to label via retrograde transport of neurotracers injected into the cerebellum, suggesting that axons of olivary neurons pass to the cerebellum in these fishes[1,7,9,25]. It remains unclear, however, how they terminate there. Some groups have theorized that all cerebellar afferents form climbing fibers1 and others have reported observing climbing-fiber-like synapses on Purkinje cell dendrites[1], whereas some Golgi preparations have failed to see them entirely[1].

Purkinje cells are the primary output neurons of the cerebellum, while inputs are received from either single climbing fibers that make multiple connections, or a multitude of parallel fibers that project to an expansive layer of dendrites[36,37]. The climbing fibers receive extrinsic information from granule cells that process this data through the cerebellar cortex. Their activity drives learning, which is established in sets such that relearning does not have to occur for intermittent behaviors[28,34,35]. Moreover, the development and maintenance of Purkinje dendrites is fueled by the trophic effect of climbing fiber signals[17]. Parallel fibers, on the other hand, determine the strength of the adaptive filter with their inputs, and contribute to the relatively large number of cerebellar neurons[1,4].

The Inferior Olive

The inferior olive is a major neural center that is found in all vertebrates[2,3,8,9,18]. Electrophysiological studies have shown that this neural center has strong neural connections with the cerebellum[1,6,7]. It is the source of climbing fiber afferents to the cerebellar cortex and induces a strong excitatory action on Purkinje cells. This connection

is believed to play a role in the neural plasticity that underlies learning mechanisms, which is contrasted by the independent pathway of parallel mossy fiber afferents[4,12,28]. While climbing fiber signals carry information related to performance results, mossy fiber impulses drive the causal activity. The activation of these separate pathways is dependent on the animal behavior. Other olivary connections include inhibitory interneurons like stellate, basket, and Golgi cells[1,6].

Cerebellar learning mechanisms are influenced by inferior olive climbing fiber afferents that induce so-called plastic changes in the cerebellar cortex[4,28]. Directed by the causal signaling provided by mossy fiber afferents, these climbing fiber afferents transmit information about the resultant effect and performance of a particular cerebellar system. This relationship underlies learned motor tasks such as adaptive modifications of the vestibulo-ocular reflex in response to extended exposure to vestibular-visual interactions[11,27]. Ablation studies are commonly employed to evaluate the source and effects of this plasticity, and recent research has suggested that these pathways may affect more than just the traditional processes predicted by the original "Motor Learning Theory[19]. Long-term potentiation and depression at a variety of locations, in conjunction with extracerebellar systems referred to as corticonuclear microcomplexes, have been shown to have profound effects on synaptic and non-synaptic plasticity with other processes like cognitive learning[6,37].

The olivocerebellar system, or tractus olivocerebellaris, accompanies the spinocerebellar tract and contains fibers that originate from the contralateral inferior olive and terminate in all parts of the body of the cerebellum[1,9,17]. In cartilaginous fishes, the inferior olive is an elongated nucleus, which occupies a paramedian position in the caudal part of the rhombencephalon and comprises small, round, and ellipsoid cells[30]. This

nucleus has been observed in a variety of cartilaginous fishes and is located in the same anatomical position as the inferior olive of the mammalian brain.

Distribution, Organization, and Morphology

Synaptic communication in the inferior olive is conducted by electrical gap junctions that are composed of Connexin (Cx) protein[19]. In mammals, this structure is entirely made up of Cx36, whereas in fish, like the zebrafish, both Cx34 and Cx35 play roles in this process[19]. These synapses are governed by the transient, electrical coupling of a variable number of neighboring neurons that ultimately give rise to the climbing fibers that innervate the cerebellar cortex. The notable heterogeneity of this particular coupling is believed to be what contributes to the complexity and unique synchronization of the inferior olive[19,28].

Dendritic arbors of inferior olive cells have some of the most complex geometry in the brain, increasing with the phylogenetic scale; however, all species that have been studied have a series of primary, secondary and tertiary branching[1,14,21]. Dendrites contain tubules, filaments, vacuoles, and some mitochondria. The olivary neurons themselves, as studied by the Golgi method and impregnation, are mostly spherical, somewhat oblong or spindle-shaped, and interlaced into a tight mesh-like cluster[1,30].

Functional Inputs

Inferior olivary efferent projections to the cerebellum are topographically localized[2,7]. They connect to the cerebellum in small bundles after crossing the midline of the medulla and coursing through the medial portion of the inferior cerebellar peduncle. After initially interposing between intracerebellar nuclei and the fibers of other afferent systems, inferior olivary axons fan outward from the midline into the cerebellar cortex. These connections then help to coordinate movement and learning behaviors[4,12].

Climbing fiber pathways to each division of the cerebellum are related to vestibular functions. The vestibulocerebellum is the part of the cerebellum where primary vestibular afferents project directly, and the flocculus is the section related to the vestibulo-ocular reflex. This portion receives visual signals relayed through the inferior olive[7,11,27].

Cerebellar action is quickly mobilized by the impulses of mossy and climbing fiber afferents, in that order, and the latter afferents also communicate information about the performance impact of the resultant cerebellar activity[1,17]. These pathways form strong synaptic connections with Purkinje cells in an intricate, ivy-like pattern[15,16,23]. Their activity is considered to be the output of the inferior olive, which affects motor behavior, learning, and even memory[12,28]. Climbing fibers have also been shown to play a critical role in synaptic plasticity, also termed neuroplasticity, particularly in cerebellar long-term depression (LTD) where excitatory synapses between parallel fibers and Purkinje cells display reduced responsiveness to glutamate[4,12].

Although there have been relatively few studies of the elasmobranch cerebellum , neurophysiological investigations, in conjuction with anatomical stuides, have revealed many overalpping elements and similar interactions between the elasmobranch and mammalian cerebellum[21,22]. The basic organization of the cerellum was evidently established early in vertebrate history[20,30]. Although it is widely held that the cerebellum plays an important role in motor control, it remains unclear how this is achieved[30,33]. For instance, profound locomotor disturbances following cerebellectomy are primarily the results of loss of postural control, and for aquatic vertebrates it is simplified due to the dense medium of their environment[30]. So why, if they perform relatively stereotyped swimming movements, is the cerebellum so well developed in some aquatic verterbrate species like elasmobranchs? One theory notes how elasmobranchs have no neutral

buoyancy and are thus required to match thrust against lift[30]. Lesion studies can be difficult to interpret because the effects can be direct or indirect to the ablation. However, some studies have shown effects to tonic muscular contractions leading to spiral swimming (i.e., asymmetry) and other navigation issues such as pectoral fin reflexes, suggesting the cerebellum plays a modulating role rather than an initiating one[30].

Main Objectives

Broadly, this study is designed to investigate the significance of the hypertrophy and complex elaboration of the cerebellar anatomy in the Atlantic stingray, Dasyatis sabina, and its relationship with the inferior olivary nucleus. Considering the complex presentation of the cerebellar corpus of the Atlantic stingray and the major role of the inferior olive in the various neural functions of the vertebrate cerebellum, a significant dynamic may exist between these two neuroanatomical structures. Our working hypothesis is that the hypertrophy and complex morphological differentiation of the cerebellum in the Atlantic stingray is the result of evolutionary forces driving an increase in neural and functional specialization. Supporting evidence for this hypothesis would be provided by an elaboration and differentiation of inputs to the cerebellum in stingrays compared to those of elasmobranchs that have a simpler cerebellar morphology. To that end, the evidence sought and examined here describes unique afferent connections from the inferior olive to the distinct cerebellar lobes of the Atlantic stingray as a potential indicator of the advanced evolutionary state of the neuroanatomical organization of the cerebellum as a whole, but particularly for the sensory processing related to the inferior olive.

Therefore, this study seeks to expand the understanding of the cerebellar structure, implicated functioning, and overall evolution to the elasmobranchs possessing a

complex cerebellar structure, with a focus on the organization of cerebellar inputs from the inferior olive. To this end, this investigation established the following aims:

1. Analyze histological sections of the stingray cerebellum in order to better characterize the anatomical organization of the inferior olive, including its various constitutional cells (e.g., neurons, glia).
2. Perform neuro-tract tracing experiments as a means of elucidating the organization of the efferent projections from the inferior olive to the cerebellum.

The evidence from these experiments and analyses should contribute to the scientific understanding of the evolutionary history of not only fish, but other organisms along the phylogenetic tree of life.

CHAPTER II:

MATERIALS AND METHODS

The research results described in this thesis are based on the analysis of 18 experimental cases that were part of a previous study on cerebellar connections.[10] Two additional experimental cases were performed to provide additional data and to confirm the observations made on the previously performed experimental cases. The care and methods involving the animals used in this study were reviewed and approved by the UHCL Institutional Animal Care and Use Committee (Protocol #s : 02.001 and 0918.002).

Field Work

The Atlantic stingrays used in this study were caught by either the researchers from the Galveston Bay system using a dip-netting method, or purchased from Galveston Bay shrimpers. For the dip-netting method, the researchers approached the stingrays in the shallows of the flats and carefully placed a plastic container with an open bottom over the animal. This rudimentary device prevented the stingray from escaping while a net was subsequently used to transfer the stingray into a bucket containing seawater. The stingray was subsequently transferred to a large ice chest containing well-aerated seawater. The insulated cooler was used for transport to the UHCL Animal Research Facility (ARF) in an air-conditioned vehicle.

Water temperature was checked prior to transport and if the temperature was over 80oF, then a sealed bag of ice could have been placed in the water in order to prevent the water temperatures from rising above acceptable limits. Transport times were less than three hours. Upon arrival, the stingrays were kept in a 700 gallon circular tank. To minimize stress to the animals, upon arrival at the facility, the fish were gradually acclimated to the holding tank temperature and salinity by incrementally replacing the

water in the transport cooler with holding tank water. Generally, use of the animals was performed within 1-1.5 weeks of arrival.

This particular stingray species was easy to catch and keep in aquarium facilities. The animals were fed weekly with peeled Gulf shrimp purchased from local bait shops. The Atlantic stingray is very resilient and could tolerate the experimental procedures for this research.

A fishing permit was required for collecting the stingrays. These permits are available at most local bait shops. There is no Texas Parks & Wildlife Department (TPWD) catch limit on stingrays. The PI was also covered under the TPWD collection permit of a colleague, Dr. George Guillen.

Surgical Procedure

The surgical preparation, neuroanatomical tracing, and histochemistry work was performed in Dr. Puzdrowski's research laboratory in the Bayou Building on the UHCL main campus,. A section of the lab was dedicated strictly to this purpose. During the times that the surgery was being performed, no other research activities were conducted in the lab. During the surgical preparation, surgical gloves and a lab coat were worn, and the surgical instruments were washed using a 4% chlorohexidine gluconate solution to minimize exposure of the animal tissue to pathogens.

The surgical methods used in the previous study10 that provided the histological materials that were analyzed in the current study are described briefly below. Stingrays of various sizes and both sexes were anesthetized with methane sulfonate (MS-222; Sigma Chemical, 60 mg/L) that was buffered with sodium bicarbonate prior to being transported to the research lab from the ARF. The sodium bicarbonate buffer neutralized the strong acidic quality of the MS-222 in order to reduce any possible pain sensations experienced by the animals. Response to a tail pinch and eye withdrawal response were

used to assess the depth of anesthesia. Surgery was not started until the animal no longer responded to these stimuli. Once deep anesthesia was achieved, the stingray was placed in the recording tank and the dorsal aspect of the brain was exposed from the level of the midbrain-diencephalic junction to the level of the posterior lobe of the cerebellum.

The surgery exposed the dorsal aspect of all three of the lobes of the cerebellum for neurotracer injection. Biotinylated dextran amine (BDA; Molecular Proboes, 3000 MW) was used for injections into the individual cerebellar lobe of interest. Neurons are particularly disposed to taking up BDA because it is a source of glucose and they readily transport it, so it makes a very suitable choice for this application. The BDA was prepared as a dry paste and placed on the tip of electrolytically sharpened 000 insect pins for 3-4 injections. To ensure complete diffusion into the tissue, BDA coated pins were held in place for 10 minutes. Finally, the incision site was sutured and the stingrays were returned to the aquatic tank in the ARF for 5-14 days before the brain was harvested and the tissue was processed.

For the two additional stingrays that were used for this research, the same surgical procedures were followed as described above with the exception that the BDA solution was filtered through a 0.22 □m filter for sterility, and that global injections into all three of the cerebellar lobes were performed.

Neuro-tract tracing

Following the post-surgical survival period, stingrays were deeply anesthetized and transcardially perfused with a 0.1M phosphate buffer and 4% paraformaldehyde-based fixative. The brains were removed from the cartilage casing and cleaned of the surrounding tissues including the membrane-like meninges, nerves, and blood vessels before being post-fixed in 4% paraformaldehyde with 20-30% sucrose to prevent ice crystals from forming in the tissue. After 24-48 hours, the tissue was sectioned into

phosphate buffer at 40 uM using a microtome and processed according to the VECTASTAIN© ABC KIT protocol from Vector Labs with a nickel-diaminobenzidine (Ni-DAB) reaction to reveal the presence of transported BDA in the cell body of the neurons projecting to the cerebellum. The nickel-diaminobenzidine reaction produces a black reaction product labeling the neurons that contain the BDA. Finally, the processed sections were mounted on chrom-alum treated glass slides, counterstained with 1% neutral red, and coverslipped.

The pattern of labeled neurons in the inferior olive following histochemical processing was visualized and analyzed with a Nikon Optiphot 2 light microscope and Nikon ACT-1 imaging software.

Histochemistry

The normal anatomy of the inferior olive was analyzed in previously processed sections of two separate brains from a previous study of cerebellar connections in the Atlantic stingray[10]. These cases were supplemented with the results from the stingrays utilized for this study.

Both brains were embedded in paraplast and sectioned at 10 um thickness. The brain of a juvenile stingray was stained using the Bodian method and the brain of an adult stingray was processed using .01% cresyl violet stain. Bodian stains are often utilized for the purpose of demonstrating nerve fibers by adding a copper metal to Protargol-S (silver proteinate), which replaces the silver in the connective tissue and allows for greater differentiation between the nerve fibers and connective tissue. This staining method provides a sharp contrast of finer structural features and morphology. Cresyl violet or Nissl staining, on the other hand, applies a cresyl violet acetate solution to stain the Nissl substance in the cytoplasm of neurons in paraformaledyde or formalin-fixed tissue in order to identify important anatomical features of neurons in the brain and spinal cord.

The histochemistry data from the neurotracer injections was visualized and analyzed with a Nikon Optiphot 2 light microscope and Nikon ACT-1 imaging software.

CHAPTER III:

RESULTS

In stingrays, the inferior olive is distinctly divided into two main neuronal clusters or regions – the dorsolateral subnucleus and the ventromedial subnucleus (*Figure 6*). The dorsolateral subnucleus is relatively smaller than its ventromedial counterpart[7,14], and, as described below, appears to have fewer overall connections with the cerebellum. The following summaries outline the results from the neuro-tract tracing and histochemical analyses.

Neuroanatomical Observations

Many of the inferior olive neurons appeared multi-polar with polygonal soma, especially of the triangular or pyramidal geometry (*Figure 7-middle/7-right*). Other cell bodies presented as more fusiform, elongated, or spindle-like (*Figure 7-middle/7-right*). Some of the stained samples provided sufficient clarity to observe the dendritic branching and axonal collaterals (*Figure 7-middle/7-right*). In the Bodian sections, the neuronal soma were estimated to range from approximately 3-5 protrusions per cell on average (*Figure 8*). These somatic and dendritic characterizations are consistent with previous observations[14]

The histochemical stains with both the Bodian and cresyl violet methods highlighted several key neuroanatomical features, from the neuronal soma to their axons and collaterals, particularly at high magnifications (*Figure 8*). The first spinal root, appearing as a long and continuously smooth texture (*Figure 9*), was initially detected as a definitive physical feature in order to orient all of the following observations. Once this neuroanatomical landmark was identified, the entire rostro-caudal extent of the elongated inferior olivary nucleus was calculated along the paramedian portion of the medulla oblongata in the rhombencephalon. In the cresyl violet staining of the adult stingray, the

inferior olive was estimated to be approximately 2.0-2.2 millimeters. The Bodian-stained brain of the juvenile stingray, on the other hand, was estimated to have an inferior olive of 1.4-1.5 millimeters in length. This discrepancy is not surprising, due to the smaller size of the juvenile stingray.

In each sample set, more specifically, the inferior olive was tracked from the entrance level of the glossopharyngeal (IXth) cranial nerve to the first spinal motor root (*Figure 9*). The ventromedial subnucleus extended this entire length, with the dorsolateral subnucleus (*Figure 10*) extending from the entrance level of the vagus (X) cranial nerve to approximately the level of the obex. Furthermore, the ventromedial subnucleus was observed as a symmetrical cluster of neurons on the ventral and medial aspects of medulla (*Figure 9*). The dorsolateral subnucleus, with similar symmetry across the midline, was observed on the dorsal and lateral aspects of this same area (*Figure 11*).

Olivo-Cerebellar Connections

The Atlantic stingray possesses a cerebellum with a unique morphology. Its structure is divided into three distinct lobes – the anterior rostral lobule, anterior caudal lobule, and posterior lobe (*Figure 1-right frame*). This tri-lobed presentation contrasts the more common bi-lobed format seen in several types of other cartilaginous fishes, such as *Raja erinacea* or the skate (*Figure 1-middle frame*). In addition to the division of the anterior lobe into two discrete lobules, the Atlantic stingray cerebellum has a high degree of foliation. Other fishes have cerebella that have been observed to present with a simple and smooth surface, like *Hydrolagus colliei* or the ratfish (*Figure 1-left frame*).

The BDA injections revealed the locations of the neurons projecting from the different parts of the inferior olive to the different lobules of the cerebellum (*Figures 13-15*). As expected, all BDA labeled neurons were found in the inferior olive contralateral

to the side of the injection into the cerebellum. In analyzing the organization of the BDA labeled olivo-cerebellar neurons in the inferior olive the first spinal root was used as a starting reference point and the sections were analyzed moving along the sagittal axis of the brain in the caudal-rostral direction. The first spinal root appeared as a distinct white band of neural tissue (*Figures 9* and *13)*. The labelled neurons were indicated by darkly stained soma (*Figures 13-15).* The locations of the olivo-cerebellar neurons projecting to each lobule of the cerebellum are described below.

Location of BDA Labeled Inferior Olive Neurons following BDA Injections into the Anterior Rostral Lobule

The anterior rostral lobule was found to receive inputs from olivo-cerebellar neurons located in the dorsolateral subnucleus of the inferior olive (*Figure 13*). It was also noted that these labeled neurons lobule were located in the more ventro-lateral part of the dorsolateral subnucleus of the inferior olive, and had a lesser rostro-caudal extent within dorsolateral subnucleus when compared with neurons labeled following tracer injections into anterior caudal lobule. Furthermore, compared to the number of labeled neurons in the inferior olive following the BDA injections into the other parts of the cerebellum, there were relatively few neurons labeled neurons following the injections into the anterior rostral lobule.

Location of BDA Labeled Inferior Olive Neurons following BDA Injections into the Anterior Caudal Lobule

Following BDA injections into this anterior caudal lobule, BDA labeled neurons were found distributed in the dorsal subnucleus of the inferior olive (*Figure 14*). It was also noted that these labeled neuronal soma were located in the more dorso-medial part of the subnucleus, and had a greater rostro-caudal extent within the dorsolateral subnucleus when compared with those labeled following tracer injections into anterior rostral lobule.

Furthermore, a greater number of neurons were labeled following the injections into the anterior caudal lobule when compared with injections into anterior rostral lobule. Of the samples analyzed, an estimated ¼ to ½ fewer cells appeared were labeled following the injections into the anterior rostral lobule than the anterior caudal lobule

Location of BDA Labeled Inferior Olive Neurons following BDA Injections into the Posterior Lobe

Injections of BDA into the posterior lobe revealed labeled neurons exclusively within the ventromedial subnucleus of the inferior olive (*Figure 15*). Among these three cerebellar lobes, the greatest number of positively labeled neurons was observed following injections into the posterior lobe. Estimations of the proportion of labeled neurons following injections into the posterior lobe compared to the anterior rostral lobule were estimated to be 1:4 and 1:2 for the anterior caudal lobule.

CHAPTER IV:

DISCUSSION

In elasmobranchs, and batoides specifically, the inferior olive is divided into a larger, ventromedial and a smaller, dorsolateral subnuclei[7,9,25]. Research in elasmobranchs with bi-lobed cerebellar structures revealed that the olivocerebellar afferents from both ventromedial and dorsolateral nuclei project evenly throughout the rostro-caudal extent of the cerebellar corpus[7]. The afferent projections from Dasyatis are more complex than those previously reported in other elasmobranchs[9]. For instance, the dorsolateral subnucleus has been shown to relate to electrosensory afferents while the ventromedial subnucleus has been associated with the lateral line system[7,36]. The former is a sense that rays utilize for hunting prey, while the latter is associated with internal balance and navigating the environment. Collectively, the olivocerebellar system produces spatiotemporal patterns that signal the climbing fibers and may contribute to neural plasticity[4,12].

The results of this study were consistent with precedent studies in terms of neuro-tract tracing pathways. Afferent projections were revealed to all three distinct cerebellar lobules from the inferior olive, including both the dorsolateral and ventromedial regions. The BDA injections projected to the lateral regions of the inferior olive. Overall, the pattern of olivo-cerebellar afferent projections in *Dasyatis* is similar to that seen in higher vertebrates. The mammalian inferior olive has been found to be divided into three main nuclei; the dorsal accessory olive, the medial accessory olive, and the principle olive[1,6,7]. Therefore, it is probable that the division of inferior olive into a two-part structure represents the primitive condition in elasmobranchs; and that the segregation of the olivocerebellar afferents within *Dasyatis* represents a parallel evolution of the olivo-cerebellar connections toward the division of the inferior olive into three separate parts.

The inferior olive projected much farther in the Cresyl violet sample set, extending nearly 1 millimeter more, due to the age difference between the individual organisms. The cresyl violet samples were of a juvenile stingray and the Bodian samples were those of an adult. This rostral-caudal size discrepancy is clearly measurable during maturation of the animal, and additional analysis could lend insight into this growth rate and progression. Greater sample sizes are required, however, to better understand the differences in this process.

In this analysis, a segregation was found in the projections from the neurons in the different parts of the inferior olive. The neurons of the dorsolateral subnucleus of the inferior olive were found to project to the anterior rostral and anterior caudal lobules, whereas the neurons of the ventromedial subnucleus were found to project exclusively to the posterior lobe. Furthermore, a segregation in the locations of the olivo-cerebellar neurons projecting from the dorsolateral subnucleus of the inferior olive was also found. The neurons in the ventro-lateral region of the dorsal subnucleus of the inferior olive were found to project to the large anterior rostral lobule, whereas the neurons in the dorsomedial region of the dorsal subnucleus were found to project to the smaller anterior caudal lobule. Also, relatively few olivary cell somata were labeled following BDA injections into the anterior rostral lobule compared to the anterior caudal lobule and the posterior lobe. The larger ventromedial subnucleus sends all of its olivo-cerebellar afferents to comparatively small posterior lobe of the cerebellum. Furthermore, following BDA injections into posterior lobe the largest number of neuronal labeling within the inferior olive was observed. It is interesting to note that the smallest lobe of the cerebellar corpus, the posterior lobe, appears to receive the greatest number of olivo-cerebellar projections from the larger, ventromedial subnucleus. The larger, anterior lobules, on the other hand, receive the fewest olivary afferent fibers, and share the smaller dorsolateral

subnucleus. Although outside the scope of this study, interestingly, afferent connections have been reported along descending pathways from the cerebellum down to regions such as the dorsal column, trigeminal, solitary, and medullary reticular nuclei in the medullary level[9]. Furthermore, fibers from the spinal column have been observed to enter the lateral aspect of the inferior olive and connections within the inferior olive itself have been shown in opossum[11].

The afferent olivo-cerebellar projections from all three of these cerebellar lobes are summarized in *Figure 16*. Of note, all of the projections are transmitted contralaterally across the midline. The funnel shapes in the figure represent how sensory stimuli are received by the inferior olive and then funneled up to the next layer of processing, the cerebellar lobules. This bottom-up processing likely includes sensory information like visual, tactile, lateral line[7], and electrosensory signals[32].

Based on these observations, there are a couple of interpretations that can be made regarding the number of cellular projections from the inferior olive to the cerebellar lobes. Each Purkinje cell in the cerebellar corpus is generally believed to receive climbing fiber inputs from only one neuron in the inferior olive. Effectively, there is a 1:1 linking of Purkinje cells to inferior olive neurons[17]. These climbing fibers, however, can contact anywhere from one to a multitude of different Purkinje cells[16,28,37]. The relatively lower number of connections observed from neurons in the dorsolateral nucleus could be explained if these neurons have axonal collaterals that are connecting with more than one Purkinje cell. These climbing fibers could be exhibiting a significant amount of divergence as connections are made with multiple Purkinje cells throughout, for instance, the anterior lobules. Alternatively, it is possible that not all of the Purkinje cells in the two anterior lobules receive inputs from neurons in the inferior olive, particularly in the larger anterior rostral lobule, as this lobule appeared to receive the fewest number of

inputs from these neurons. However, this second interpretation is unlikely considering the integral role that climbing fiber inputs play in the functioning of the Purkinje cells. The uncertainty of these climbing fiber synapses and terminal patterns was also reported in other studies[11]. It is more probable that the neurons projecting to the anterior lobules of the cerebellar corpus are doing so via numerous diverging climbing fiber collaterals.

Similarly, the histochemical analysis corroborated previous observations[13]. Both the Cresyl violet stain and the Bodian method effectively illuminated the structural features of the neurons of the inferior olive. The soma mostly presented as primarily polygonal but also some appeared pyramidal. Dendritic extensions numbered 3-5 per cell, in addition to extensive axons. The variety seen in this dendritic arbor, with some highly ramified and others not, has also been observed in the mammalian and avian inferior olive[14]. This similarity implies that the inferior olive of cartilaginous fish described here may represent an earlier evolutionary form.

The inferior olive is one of the major pre-cerebellar nuclei. The olivocerebellar system, both its structure and function, has been studied extensively in mammals, and has been well-reported as homologous to that in chondrichtyes[7,13]. While the inferior olive in the latter is divided into only two subnuclei, the inferior olive in the former is organized into three distinct cell masses – the principal, medical accessory, and dorsal accessory nucleus. Additionally, the cerebellum in elasmobranchs does not include basket cells or the definitive inhibitory network established by the axon collaterals of Purkinje cells as is observed in the mammalian counterpart[21]. The topographical organization of medial and lateral projections from the inferior olive to the molecular layer of the cerebellum has been shown in guitarfish to resemble that of mammals[7,31]. Although direct correlations between these two homologous structures have not been established, this system is believed to be responsible for the coordination of motor learning and control by the

cerebellum. Our results describe the morphological characteristics and neural connections of the inferior olive with the cerebellar lobules of a stingray species. These insights increase the resolution of established knowledge about the evolutionary history of Chondrichthyes. In order to truly understand the evolution of the cerebellum, this complex structure should be compared with different animals across various species. Perhaps the conclusions reached about the role of the morphological differentiation in the stingray cerebellum will inform that of higher organisms like humans.

Overall, the results from both the histochemical and neuro-tract tracing aspects of this study provide detailed descriptions of both the dorsolateral and ventromedial nuclei of the inferior olive. The neurons of these distinct regions were visualized by the chosen staining methods to the extent that they could be accurately characterized by a variety of physical features, from soma shape to dendritic branching. The neuro-tract tracing data linked the nuclei of the inferior olive to specific cerebellar lobules in a structural scheme that appears suggest an order of organization. As has been discussed, these key observations are reinforced in the precedent literature in terms of the physical characterizations and neuroanatomical connections of the inferior olive.

These olivocerebellar connections have been described in the Atlantic stingray, which is an advanced cartilaginous fish in terms of its uniquely complex cerebellar morphology. The evidence provided here adds to the increasing body of knowledge that suggests these relatively sophisticated neuroanatomical connections are due to functional specializations of behavior in these animals. Subsequent research studies would further define and contextualize these observations in the larger scope of evolutionary progress from fish to mammals. Such experiment designs and their aims are suggested in the following section.

CHAPTER V:

CONCLUDING REMARKS

Future Directions

The results of this proposal could indicate the direction of a number of future research endeavors. Depending on the specific cerebellar regions highlighted by this project, another experiment could be designed to further elucidate the particular neuronal pathways associated with these areas. This hypothetical research could be modeled as an electrophysiological study in order to determine the origin of particular sensory stimuli. Recordings could be taken in the target areas of the cerebellum (e.g., anterior lobule), or the cerebellar projections themselves (i.e., inferior olive), and the applicable connections would be implied. The neural network revealed may guide further investigations, from population coding to learned behaviors.

As a supplementary follow-up proposal, an ablation study protocol could be devised in which select neuronal pathways, or entire cerebellar lobes, are severed in anticipation of marked changes in cerebellar function. These specific connections would be chosen based on present indications of involvement in cerebellar processes, like which afferent nuclei input to which cerebellar lobe. The intended outcome of this project would be to establish a more complete functional map of the complex tri-lobed cerebellum in cartilaginous fishes, with an emphasis on better understanding the integrative operations of the brain.

Surveying the implications of the anatomical asymmetry of the cerebellum, albeit tangential to the previously suggested inquiries, could also be a very valuable examination. Approximately half of the cerebella observed in Atlantic stingrays have been reported as manifesting a right-sided morphology, in which the anterior caudal lobule is located on the right side of the midline, whereas the other half appear to

distribute about evenly between a left-sided morphology and an intermediate type morphology (*Figure 17*). Electrophysiology experiments could be performed in order to identify sensory projections and/or cerebellar connections that may differ depending on the anatomical symmetry of the cerebellum. Possible patterns might indicate a relationship between cerebellar symmetry and corresponding function.

In light of evidence suggesting that, across mammalian species, the ratio of neurons in the cerebral cortex is consistent with those in the cerebellar cortex, regardless of cerebral volumetric growth10, another experiment could be devised to study the relationship between cerebellar connections with cerebra of various sizes. The hypertrophy of these two core structures of the nervous system may not progress in parallel in terms of overall volume, but the density of neurons in the cerebellar cortex appears to maintain a ratio of 3.6:1 with the cerebral cortex neurons. This cellular synchronization suggests a fundamental importance to the connectivity of their respective compositions. Further investigations could compare fish with fish or fish with mammals in an effort to qualify this connective correlation, from olivary cells and climbing fibers to Purkinje cells and their synapses.

The categorical purpose of this field of research is to provide a greater knowledge about the evolution of animals. This proposal focuses on one of the supposed sources of all terrestrial life – marine organisms. In order to truly understand the evolution of the cerebellum, this complex structure must be compared with different animals across various species. Stingrays, along with other similar cartilaginous fishes like skates and sharks, should be matched against other dissimilar aquatic animals like rat fishes or ghost sharks. Perhaps the conclusions reached about the role of the morphological differentiation in stingray cerebellum will inform that of higher organisms like humans.

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IMAGES

1. Benton, M.J. (2005). Evolution of Cartilaginous Fishes. [photograph]. Retrieved from https://upload.wikimedia.org/wikipedia/commons/f/fd/Evolution_of_cartilaginous_fishes.png.
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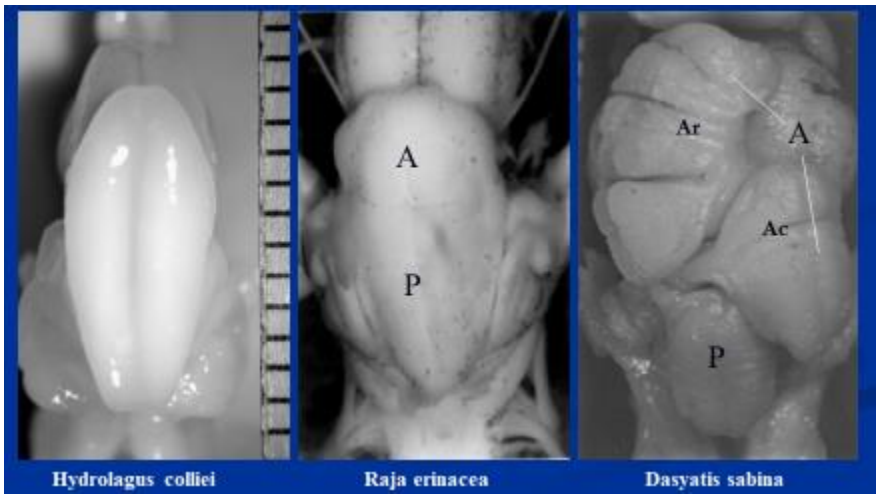
APPENDIX A:

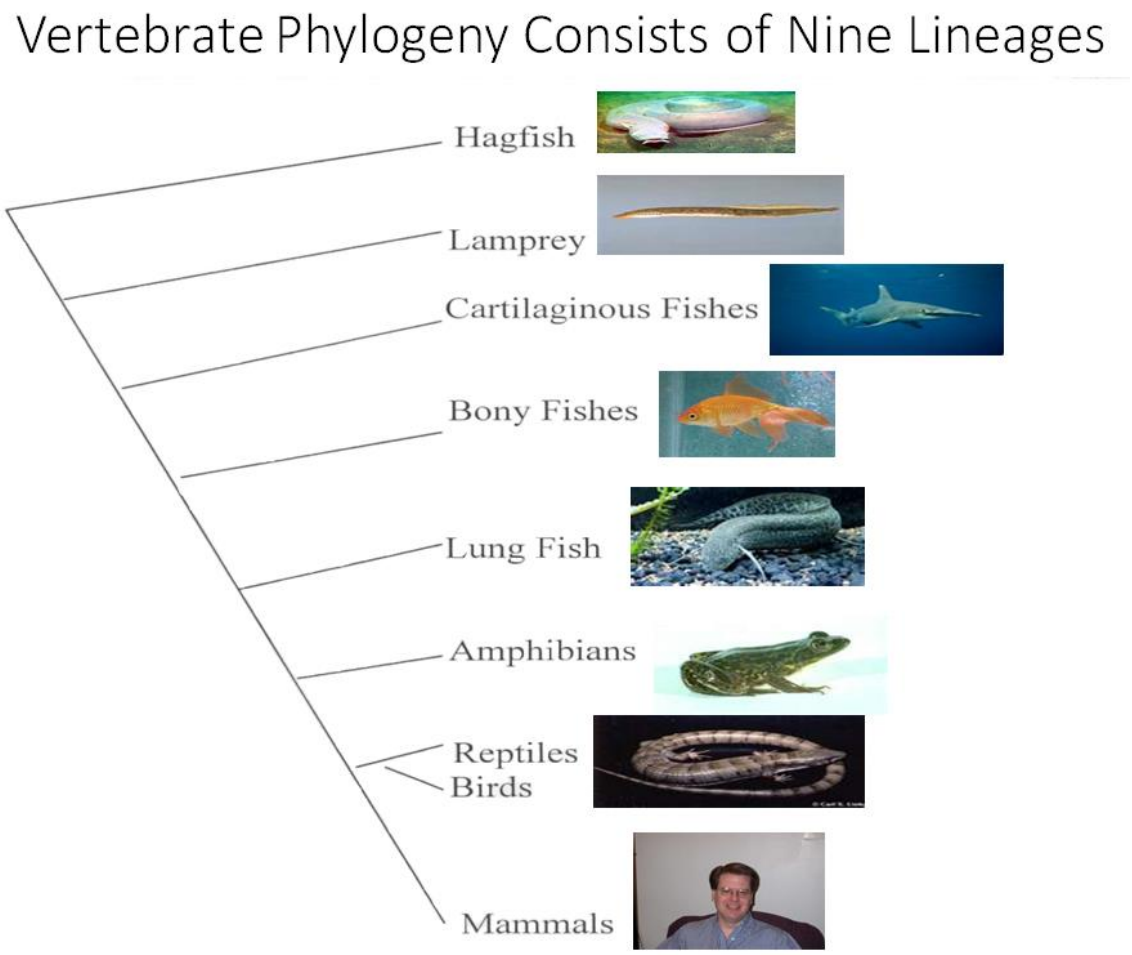
FIGURES

Figure 1. Comparative Stingray Cerebellum Anatomy (Puzdrowski, 2013)



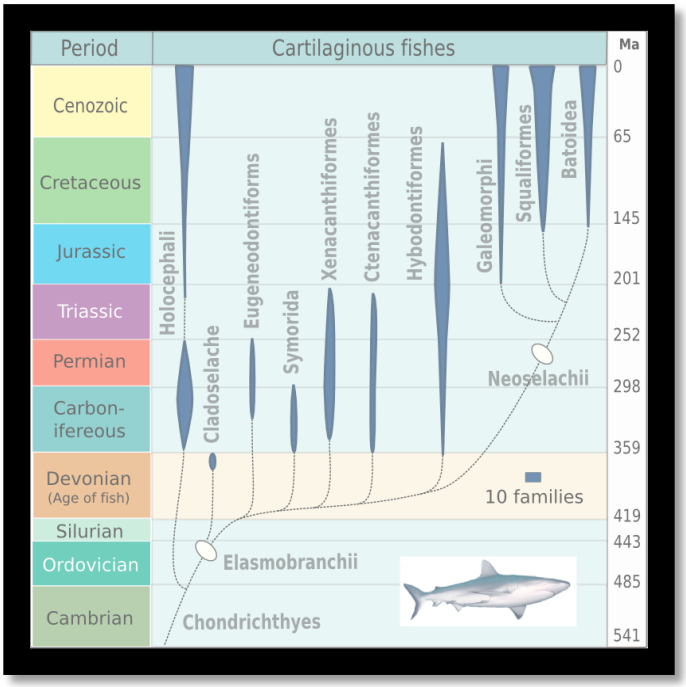
The corpus of the cerebellum in cartilaginous fishes exhibits a wide range of complexity: from a smooth, unfoliated structure in ratfish (Hydrolagus), to a bilobed structure as in skates (Raja), to a multilobed, highly foliated structure as in stingrays (Dasyatis). In skates and stingrays, the corpus is divided into an anterior lobe (A) and a posterior lobe (P). The anterior lobe in stingrays, however, is further divided into a rostral lobule (Ar) and a caudal lobule (Ac).

Figure 2. Phylogeny of Vertebrates – Fish Lineages



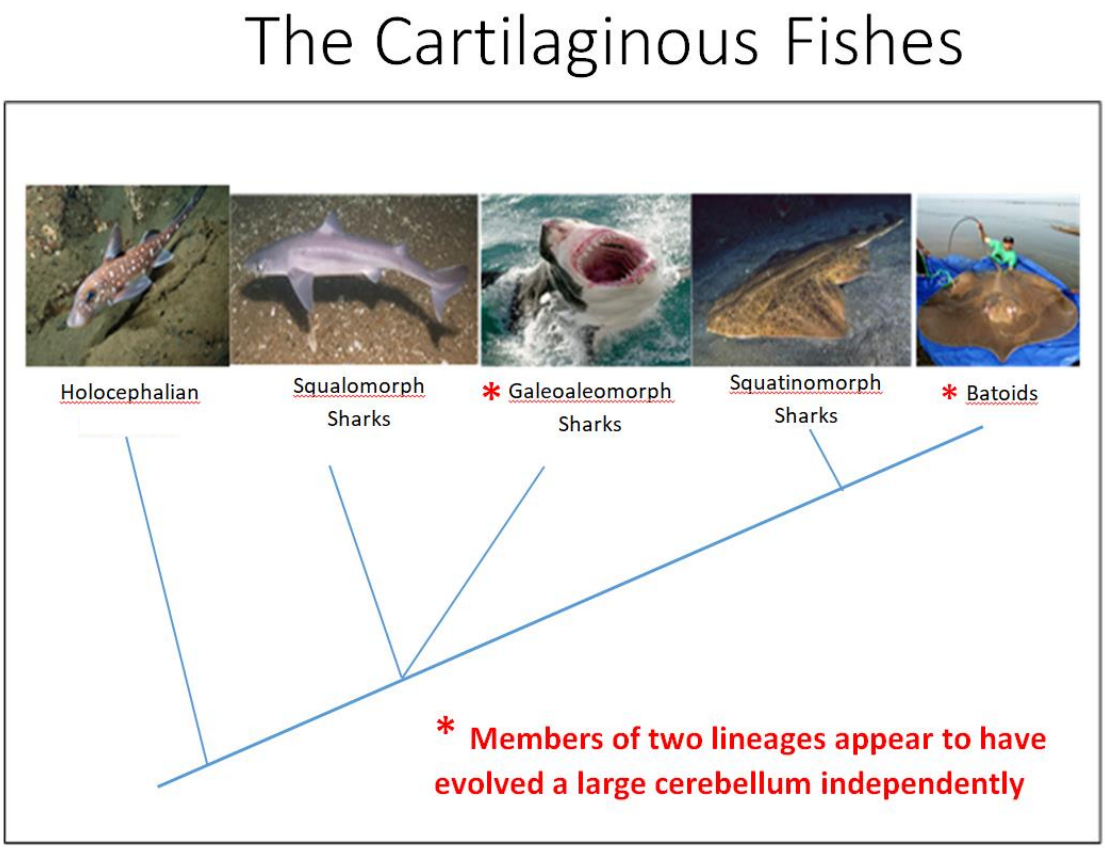
Vertebrates are comprised of nine distinct lineages, as illustrated by this phylogenetic tree. Stingrays belong to the Cartilaginous Fishes lineage, which forms the root node of the monophyletic group, or clade, consisting of bony fishes, lung fish, amphibians, reptiles, birds, and mammals. The vast majority of fishes belong to the Bony Fishes lineage, while the remaining members are divided among Hagfish, Lamprey, Cartilaginous Fishes, and Lung Fish.

Figure 3. Evolution of Chondrichthyes[1]



This evolutionary timeline shows the developmental history of Chondrichthyes, or cartilaginous fishes, over the past 540 million years. The Atlantic Stingray, and other stingrays, belong to the Batoidea superorder of the Elasmobranchii subclass. Beginning approximately 400 million years ago in the Devonian Period, also known as the Age of Fish, their group is comprised of nearly 1,000 living species. Their fossil record is more established due to their cartilaginous endoskeleton, thus their ancestral phenotypes, particularly the bony mandible of gnathostomes, are well described. Fostering the knowledge about their unique morphologies will facilitate a greater understanding about the evolution of Chondrichthyes.

Figure 4. Phylogeny of Chondrichthyes

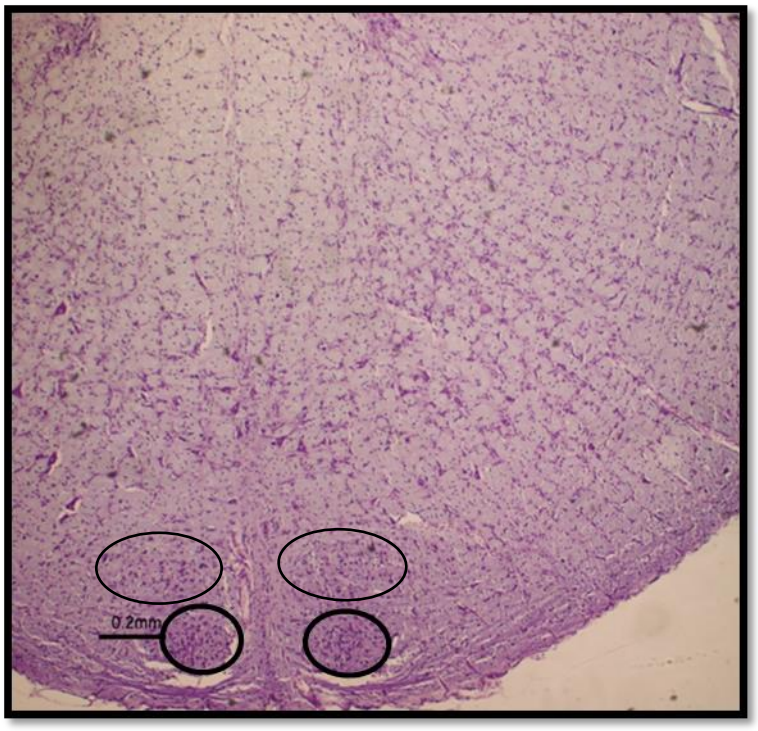


This phylogenetic tree of the extant cartilaginous fishes, or Chondrichthyes, highlights how both the galeoaleomroph sharks and the batoids, of which stingrays are members, possess a large cerebellum. The squatinomorph sharks, on the other hand, do not present a similarly enlarged cerebellar corpus, despite sharing a common ancestor with the batoids. This distinction suggests that galeoaleomorph sharks and batoids evolved their cerebellum independently.

Figure 5. Atlantic stingrays

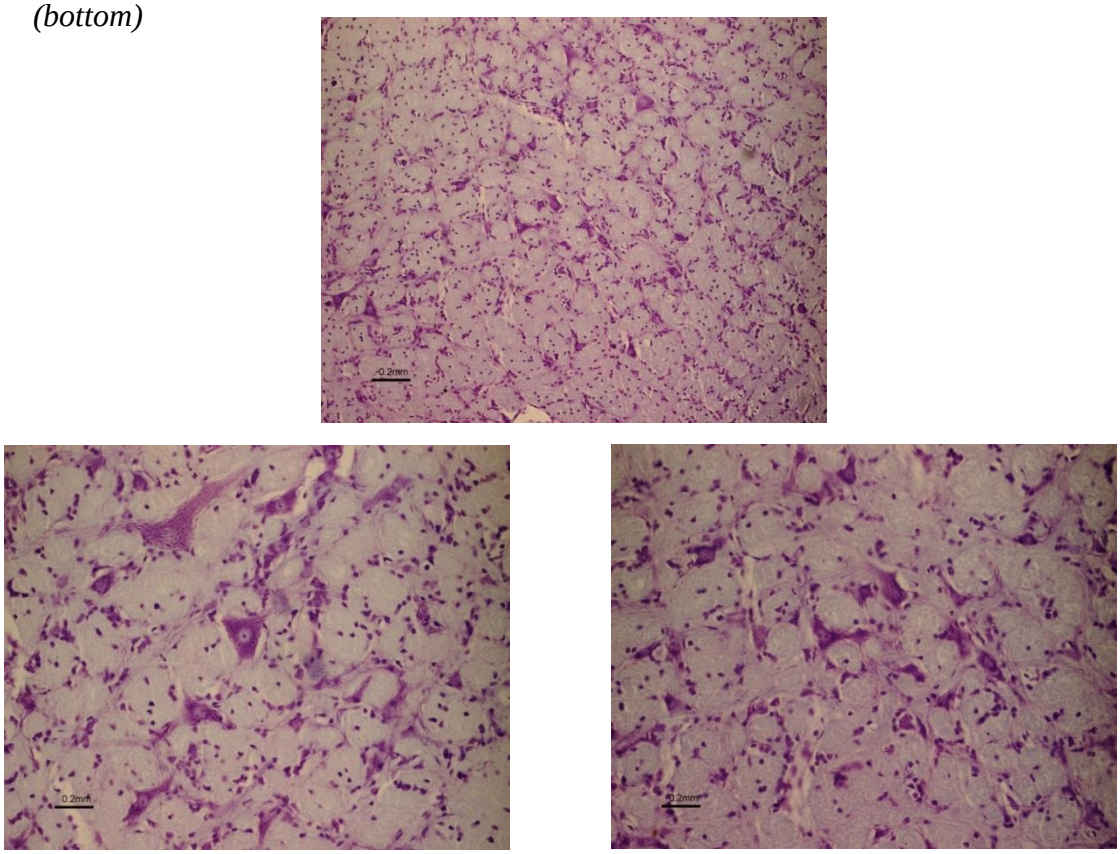
The stingrays used in this research were housed in the UHCL Animal Research Facility (ARF) for up to 1-2 weeks, where they were inspected daily for signs of distress and general health. These two specimen, one female (left) and one male (right), are distinguished by the presence of the clasper copulatory organs at the base of the tail in males.

Figure 6. Inferior Olive, Ventromedial and Dorsolateral Subnuclei, Cresyl Violet, 10X



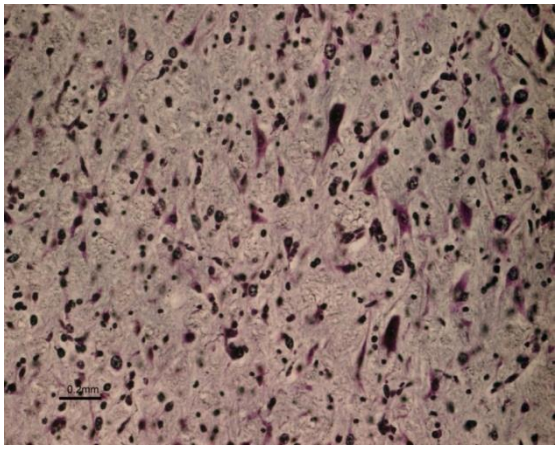
The ventromedial (bold border) and dorsolateral (light border) subnuclei of the inferior olive are visible along either side of the midline.

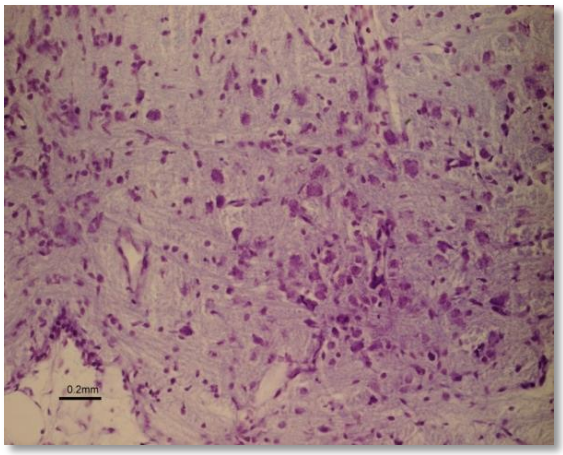
Figure 7. Inferior Olive, Dorsolateral Subnucleus, Cresyl Violet, 10X (top) to 20X (bottom)

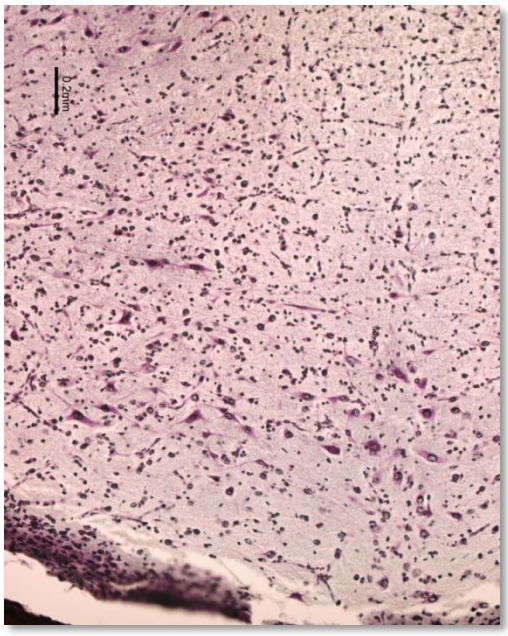


The neurons of the inferior olive neurons present in a variety of morphological shapes and sizes. Some are multi-polar with triangular soma, as shown in the middle of this figures, while other cells bodies appear as more fusiform, elongated, or spindle-like, as shown in the bottom-right portion of these frames.

Figure 8. Inferior Olive, Ventromedial Subnucleus, Bodian 20X (left), Cresyl Violet 10X (bottom), Cresyl Violet 4X (right)

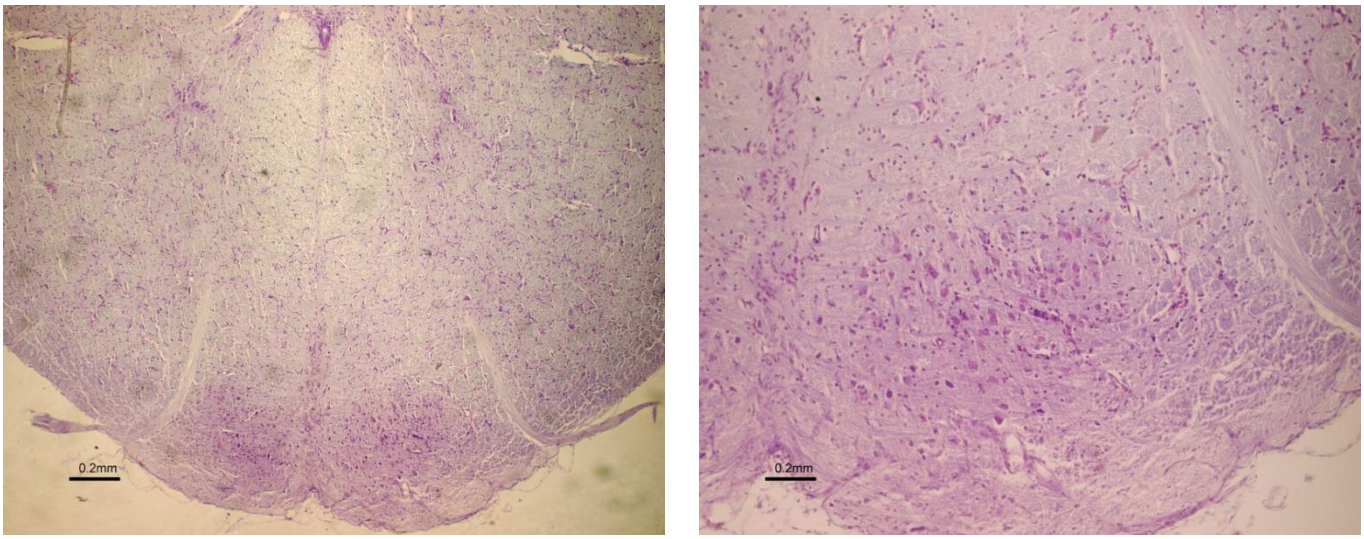






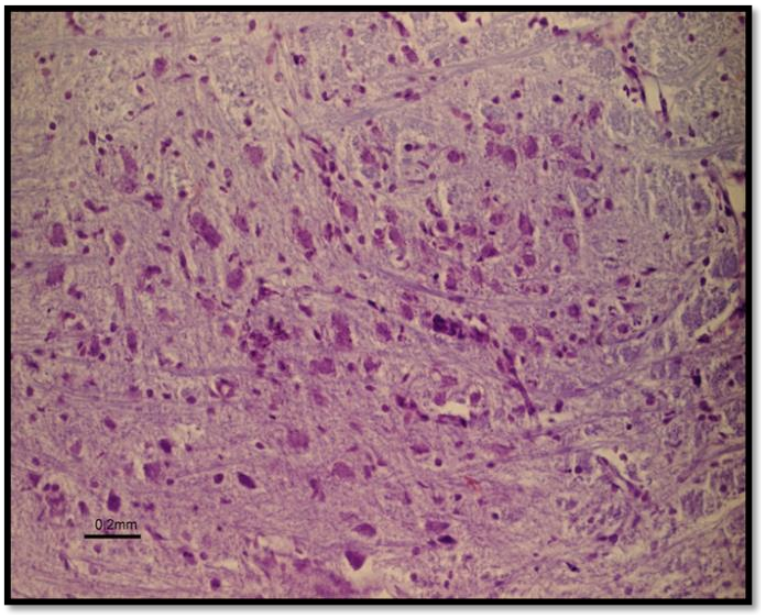
In addition to the variable somatic geometry displayed in Figure 7, these sections also provided exceptional clarity of the dendritic branching from these inferior olive neurons. Approximately 3-5 protrusions were observed per neuron on average. These characterizations of both the soma shapes and the dendritic branching are consistent with previous observations by Iwahori and Kiyota (1987) in the red stingray.

Figure 9. Spinal Root, Cresyl Violet, 4X (left) and 10X (right)



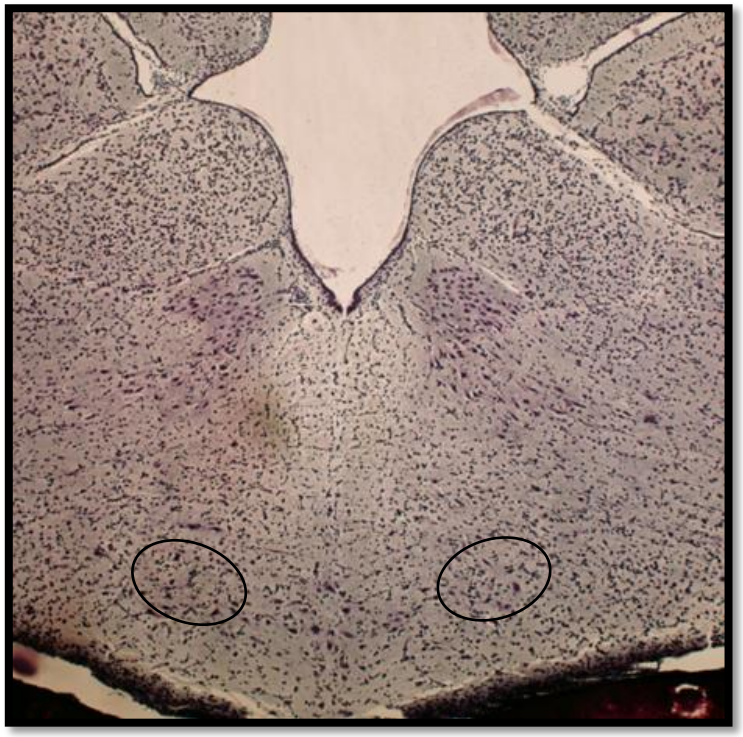
The ventromedial subnucleus of the inferior olive is visible to the left of the first spinal root in this frame.

Figure 10. Inferior Olive, Dorsolateral subnucleus, Cresyl Violet, 20X



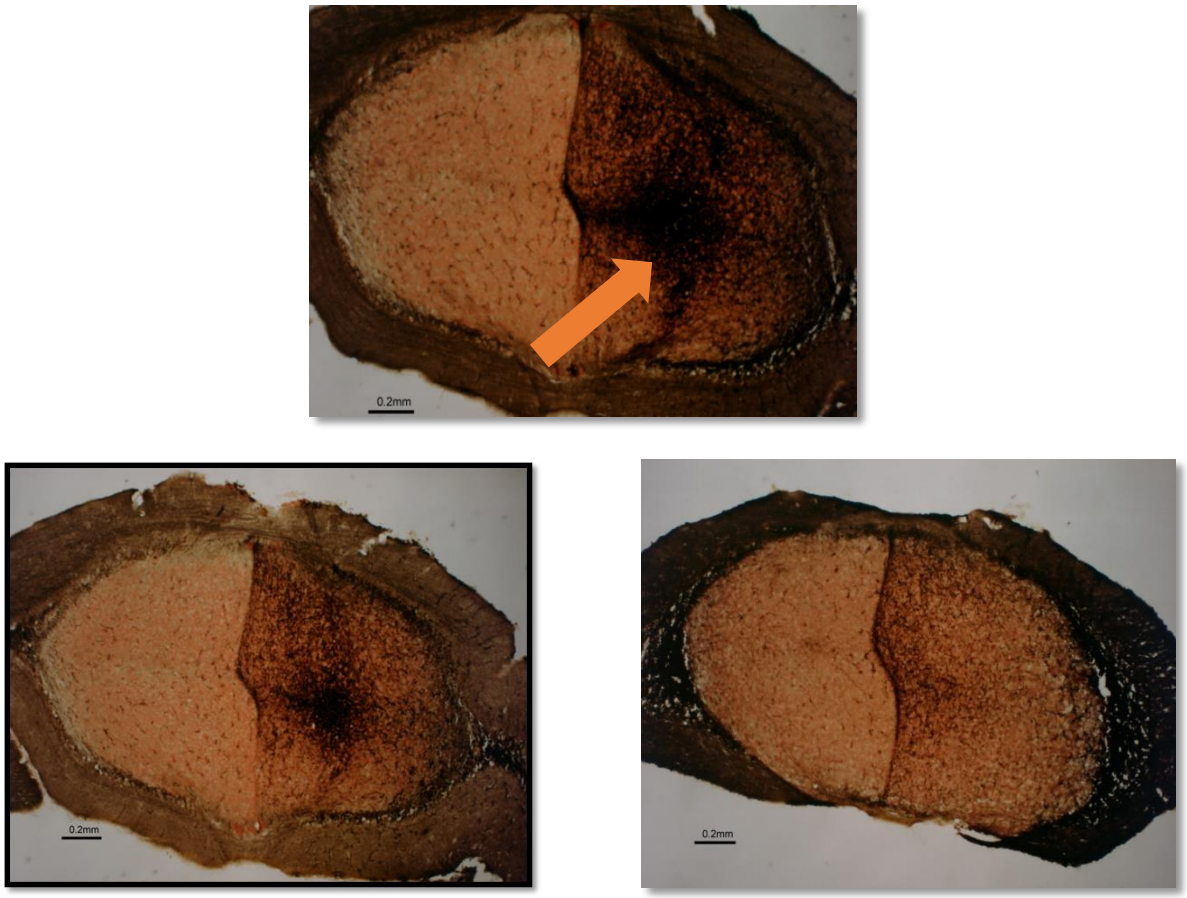
Like the ventromedial subnucleus, the dorsolateral subnucleus of the inferior olive is symmetrical across the midline and contains several cell shapes, such as these more rounded soma.

Figure 11. Inferior Olive, Dorsolateral, Bodian, 4X



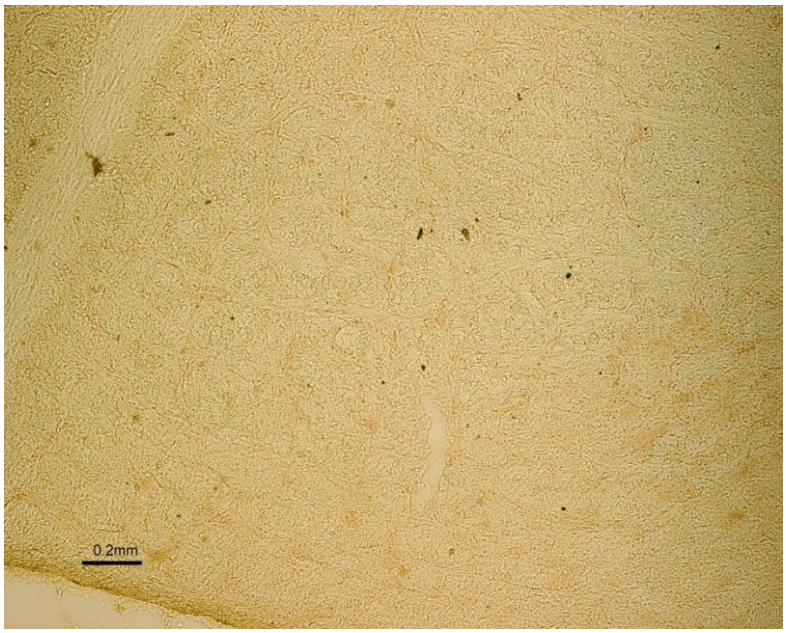
This Bodian section features symmetrical neuronal populations of the dorsolateral subnucleus, as indicated by the encircled regions.

Figure 12. BDA injection site, posterior cerebellar lobe



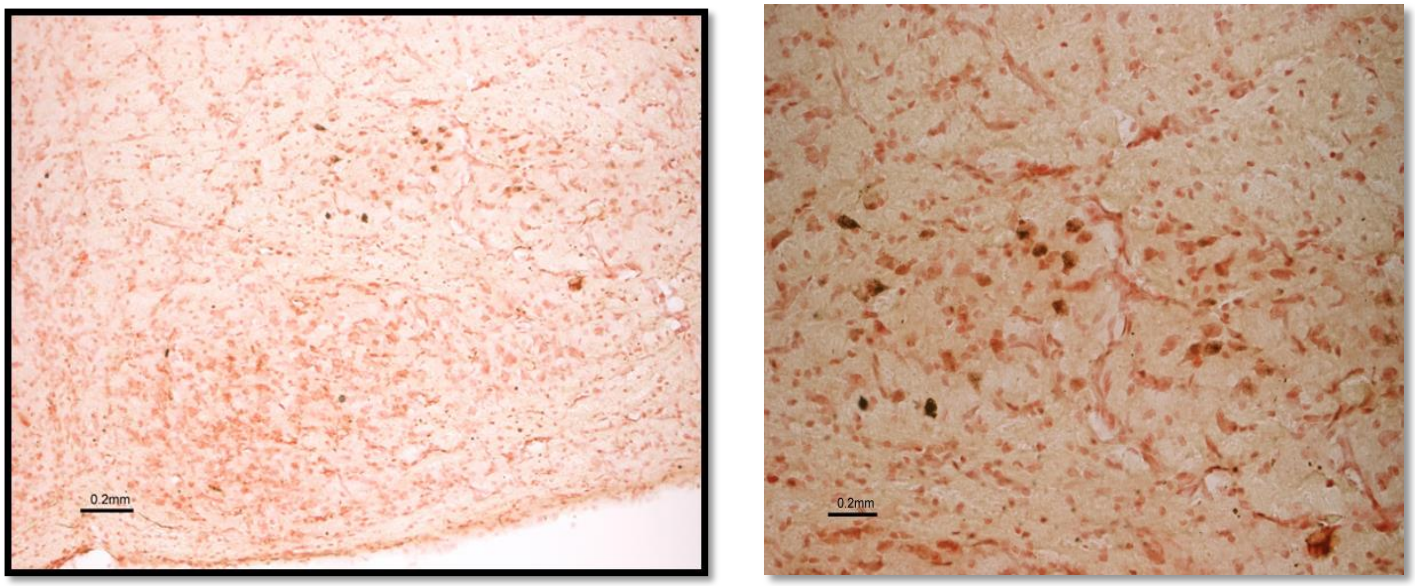
This series of images show how the BDA is transported by the neurons of the cerebellum to those of the inferior olive via retrograde transport over time (top to bottom left and right). The arrow in the first frame indicates the initial BDA injection site, which was contained to just one hemisphere in this posterior lobe.

Figure 13. Anterior Rostral Lobule BDA Injection, 10X



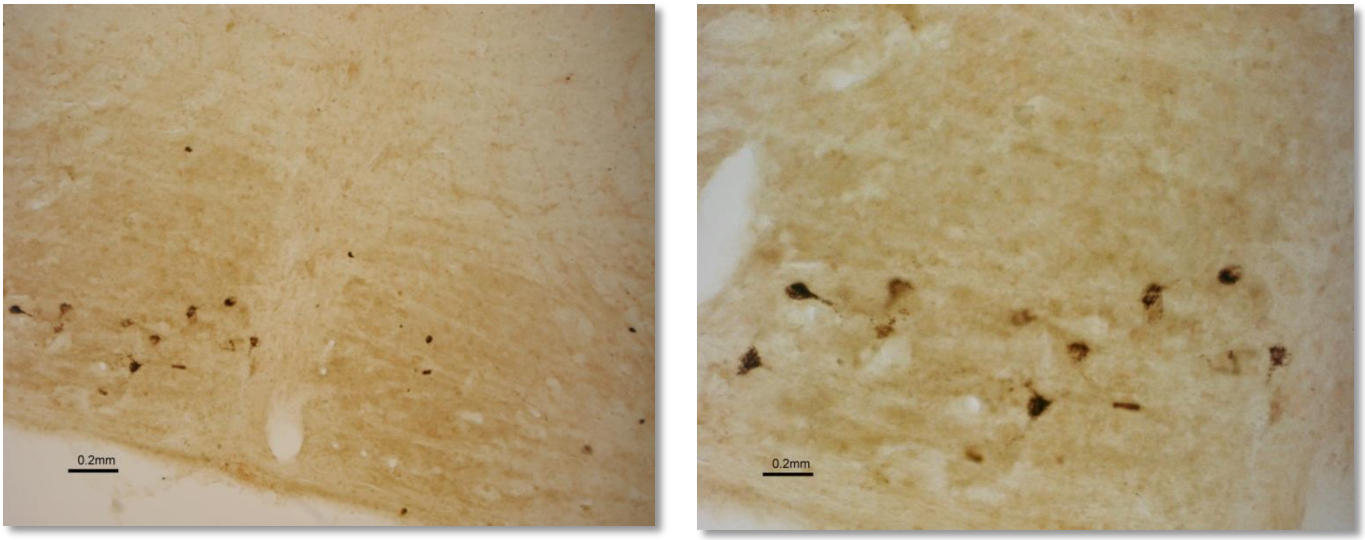
On the left side of the left frame, the spinal root can be seen with a few BDA-labeled neurons in the lateral portion of the dorsolateral subnucleus following the neuro-tract tracing injection into an anterior rostral lobule.

Figure 14. Anterior Caudal Lobule BDA Injection, 10X (left) and 20X (right)



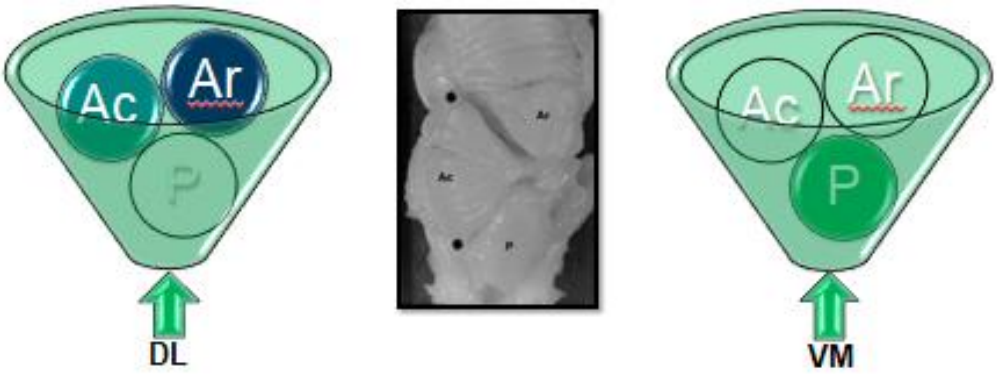
After BDA injection into the anterior caudal lobule, labeled neurons were observed in the medial portion of the dorsolateral subnucleus. More cells were observed projecting from this anterior caudal lobule compared to the anterior rostral lobule. In the right frame, the variety in the cellular morphologies of these BDA-labeled neurons is on display, from elongated to triangular soma.

Figure 15. Posterior Lobe BDA Injection, 10X (left) and 20X (right)



More cells projected to the ventromedial subnucleus than either of the two anterior lobules following BDA injection into the posterior lobe. These cells were uniformly distributed, as shown in these two frames.

Figure 16. Summary diagram of connections in the inferior olive



Schematic of contralateral connections between the DL and VM regions of the IO and the cerebellar lobes of DS: Anterior rostral (Ar); Anterior caudal (Ac); Posterior (P). Projections represented by solid color fill.

Figure 17. Atlantic Stingray Cerebellar Morphology Types[2]

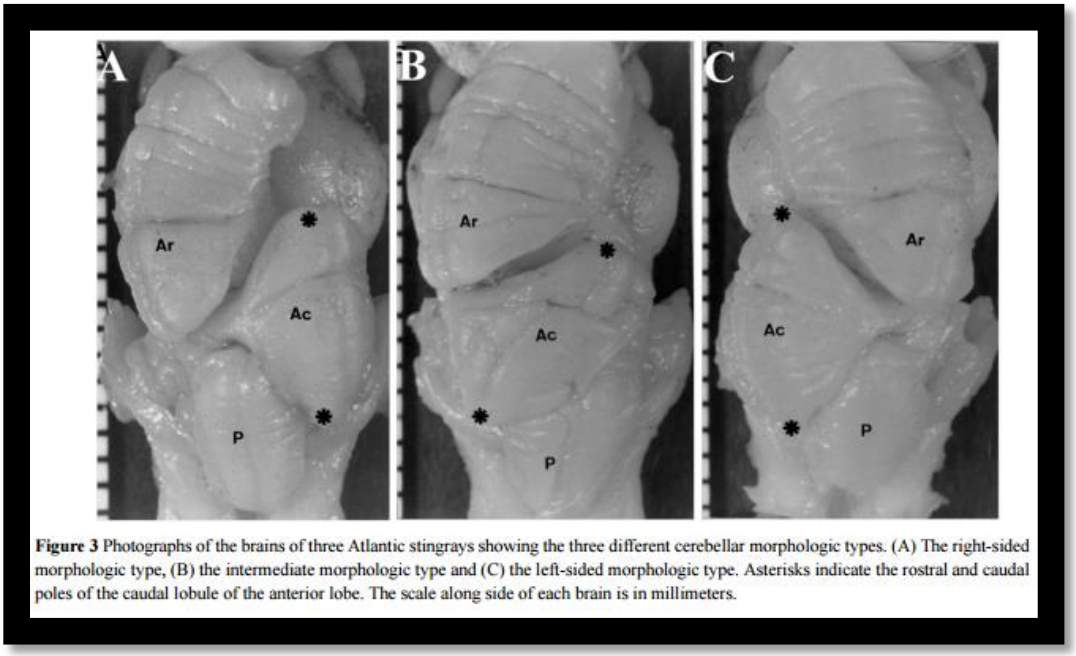


Figure 3 Photographs of the brains of three Atlantic stingrays showing the three different cerebellar morphologic types. (A) The right-sided morphologic type, (B) the intermediate morphologic type and (C) the left-sided morphologic type. Asterisks indicate the rostral and caudal poles of the caudal lobule of the anterior lobe. The scale along side of each brain is in millimeters.

These photographs illustrate the different cerebellar morphological types observed in Atlantic stingrays. Approximately half of the cerebellum observed in stingrays have been reported as manifesting right of the midline, whereas the other half appear to distribute about evenly between asymmetrically left and intermediate types[25]*. BDA injections were made into all of the three cerebellar lobes, irrespective of the morphological type.*