THE EFFECT OF SOCIAL EXPERIENCE ON BRAIN MORPHOLOGY IN ZEBRAFISH (DANIO RERIO)

by

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I. Abstract

Adult male Zebrafish form stable dominance relationships that consist of dominant and subordinate animals. The long-term effects of social dominance on nervous system function remain poorly understood. Here, we investigated how social stress affects the morphological architecture of the hypothalamic dopaminergic nuclei implicated in social regulation and aggression. The dopaminergic system is a prime target of social factors given dopamine's (DA) involvement in aggression, depression, stress and anxiety. We tested the hypothesis that social dominance induces morphological reorganization of the hypothalamic dopaminergic Posterior Tubercular rostral (PTar) and Caudal (PTac) nuclei. Both nuclei receive visual and olfactory social cues and relay the integrated information to spinal cord locomotor circuits to modulate locomotor activity. To test the effect of social dominance on the number of DA neurons we used the transgenic zebrafish line *Tg(dat:EGFP)* that expresses EGFP in all CNS dopaminergic neurons. Using confocal imaging of brain slices coupled with digital analysis, we compared the number of DA neurons in the PT nuclei between dominant and subordinate animals after two weeks of continuous social interactions; while group-housed (6 communally housed males) served as a control group. Our results show a significant increase in the number of DA neurons in dominants compared subordinate animals, while the number of DA neurons in communals was not statistically significant from neither dominants nor subordinates. Our results demonstrate that social dominance induces morphological reconfiguration of the hypothalamic DA system in a social status-dependent manner, and likely to have broader implications on other vertebrate social species.

II. Introduction

In vertebrate animals, the hypothalamus is located dorsally to the anterior pituitary and ventrally to the thalamus.[1] It is structurally composed of several nuclei and also many interconnected neuronal populations.[1] The neurons in the hypothalamus are important for regulating fundamental bodily functions such as social behavior, movement, stress, hunger and metabolism, sleep, and blood pressure, among other things.[2] The hypothalamus has been studied previously in many different contexts, including social regulation such as aggression. The hypothalamus integrates internal and external sensory signals, processes them, and exerts regulatory signals.[1] This is achieved by two primary modes of action which operate in parallel

within the hypothalamus: secretion of messengers from the neurons into the surrounding tissues and also direct innervations with synaptic neurotransmission.[2] The hypothalamus is the master homeostatic regulator for a wide range of physiologic processes, and it is composed of functional cell types conserved throughout many species, including vertebrates, such as zebrafish, rodents, and humans.[3] Dopamine is a neurotransmitter which is essential to the central nervous system. The dopaminergic systems are known to contribute to control of motor activity, perception, and behavior.[4] Dopamine is known to be widely distributed in the brains of vertebrate animals and it is essential to cognition, movement, and endocrine responses.[5] Dopamine is of interest to this study because it is implicated in regulating social aggression, anxiety, depression, and motivation in many animal species. The hypothalamus contains numerous dopaminergic nuclei, and the areas of interest in this study were the PTN, which is analogous to the A11 in mammals, the A10, the pvopA, and the pvopB.

The dopaminergic neurons associated with the descending diencephalospinal system are located in the PT, or posterior tuberculum, in zebrafish.[6] This region corresponds to the A11 group in the hypothalamus and thalamus in mammals.[6] There is vast similarity between the general anatomy and organization of the hypothalamus in all vertebrate animals.[3] There is a dopaminergic innervation of the spinal cord which is thought to originate from the A11 within the diencephalon, which houses the hypothalamus.[7] The A11 neurons have a unique morphology in that they are especially large and have obvious, long axonal projections. The A11 system in vertebrates is known to provide the sole dopaminergic innervation of the spinal cord and hindbrain, and has been implicated in the modulation of sensory processes and locomotion.[8] However, the mechanism of how this system's dopaminergic activity contributes to the motor behavior and sensory stimuli of vertebrate animals remains unclear.[8]

The neurons within the pvopA and pvopB region of the hypothalamus have the morphology of small circular cells which are clustered together and do not appear to have any axonal projections. These cells could be involved in the regulation of homeostasis in the vertebrate animals, as a result of the hypothalamus being a key regulator of homeostasis.[1] The hypothalamus is comprised of several nuclei whose neuronal populations produce multiple neurotransmitters and neuropeptides, which regulate fundamental bodily functions such as temperature control, metabolic rate, sexual behavior and reproduction, thirst and hunger,

emotional responses, and circadian rhythm.[1] As a result of these cells not having any projections to the spinal cord, it is plausible that pvopA and pvopB may be some of the nuclei involved in homeostatic regulation of the vertebrate.

It has been postulated that the dopaminergic neurons within the A10 region are a recent acquisition in the mammalian and avian brain.[2] The A10 neuronal morphology is most similar to the A11 morphology in that some long axonal projections are also present, which is different than the pvopA and pvopB regions which have no visible axonal projections. It is not known for certain the interactions of the A10 neurons with the spinal cord, however, there are some similarities in dopamine and glutamate release between the A11 and A10 neurons.[7] This suggests that the A10 could also be involved in locomotion with axonal projections to the spinal cord, comparable to the A11 neurons.

In a wide variety of vertebrate species including zebrafish, mice, and rabbits, there are dopaminergic nuclei which send long axonal processes to the spinal cord.[7] The A11 neurons especially, have long-range projections to the brainstem, spinal cord, and telencephalon which have been linked to pain modulation and spinal locomotor networks.[8] These projections are thought to play a part in the locomotion of these vertebrate species by innervating the Mauthner neurons within the spinal cord. The Mauthner neurons are a pair of large reticulospinal neurons which are command-like neurons that regulate the startle response fish demonstrate due to auditory stimuli.[9] The startle behavior is an essential audiomotor function for animal survival and its occurrence can be modified by environmental stimuli.[9]

The vertebrate animal *Danio rerio*, or zebrafish, is the model organism for this study. When two male zebrafish are paired together, they develop a social relationship where one fish becomes dominant and the other submissive. Through previous experiments, it has been demonstrated that the social status the fish develop is evident through a change in swimming behavior where the dominant fish swims more than the submissive fish. The social status is also manifested through attack/retreat behavior where the dominant animals attack more and retreat less, and the submissive animals attack less and retreat more. The question of interest is whether or not there is a physiological mechanism to the shift in locomotor behavioral change due to the zebrafish social status and social relationships. The several hypothalamic areas mentioned are well characterized in their physiological roles, which allows for investigation of functional and

developmental regulation.[1] Any changes in zebrafish locomotor behavior could be due to morphological changes within the A11 region in the hypothalamus, or any other hypothalamic dopaminergic nuclei system which projects to the spinal cord and regulates locomotor activity. Zebrafish are prominent and important model systems for the physiological and behavioral aspects of neural development.[2] They are vertebrate organisms that are responsive to social manipulations, which makes them a valuable vertebrate model organism for studying hypothalamic dopaminergic nuclei morphogenesis.[2]

Therefore, the research interest is whether the morphology of the dopaminergic nuclei in the hypothalamus are socially dependent. The hypothesis is that social status affects the dopaminergic nuclei which regulate motor behavior. If the hypothesis is correct, then there would be an increase in the number of dopaminergic nuclei in the dominant fish and a decrease in submissive fish.

III. Materials and Methods

Isolation and Pairing

This study involved the use of a transgenic zebrafish line *Tg(dat:EGFP)* which expresses enhanced green fluorescent protein (EGFP) in all the dopaminergic neurons. Male zebrafish were isolated for seven days. The isolation involved placing fish in the same size tank with dividers on either side to ensure that the isolated fish could not see or interact with any other fish. Once the isolation period ended, male zebrafish of the same relative size were paired together for a fourteen-day period. During this pairing period, the zebrafish began developing social relationships. Through observation it became evident which zebrafish was dominant and which was submissive. As soon as two fish were paired together in the same tank, they began to fight for social dominance by biting and attacking each other. Very soon, a relationship was formed where the submissive fish would stay near the bottom corner of the tank and would barely swim, and the dominant fish would swim all throughout the tank and would constantly nip at the submissive fish to ensure dominance. Throughout the fourteen-day pairing period, the fish were observed periodically to ensure that the social relationship remained the same throughout the pairing process. The fish were identified via different morphological traits they may have had such as a stripe break in their pattern or if they had a slightly longer/shorter tail fin.

Extraction and Fixation

Once the fourteen-day period was over and the fish had been cemented in their social position, dominant or submissive, the fish were placed into separate, labeled containers and euthanized in a heavy dose of MS-222 (0.2% by volume). Then, the brains of the zebrafish were carefully extracted to ensure that the area of interest, the hypothalamus, remained intact. Once extracted, each brain was placed in microcentrifuge tubes along with approximately 1.0 mL of 4% paraformaldehyde in phosphate-buffered saline (PBS) to begin the fixation process. The tissue would then sit overnight at 4° C on a stir plate. The following day, the tissue samples were washed in 4 separate washes with 0.1% triton in phosphate buffered saline (PBS-T) for 25 minutes each wash. After the last PBS-T wash, 1.0 mL of a cryoprotectant, 30% sucrose in PBS, was added to the tissue samples and was placed once again at 4° C overnight to ensure that the samples were prepared for freezing. The fixation process is essential to keep the tissue of the sample intact during the following experimental procedures.

Freezing

The fixed samples were then taken from the microcentrifuge with cryoprotectant after the 24-hour period and the freezing process was started. A base mold was filled halfway with optimal cutting temperature compound (OCT). The brain sample was then removed from the sucrose cryoprotectant and was dried as much as possible with a kimwipe without harming the tissue. The sample was then placed into the OCT in a sagittal position as shown in figure 1 with the letters R, D, C, and V demonstrating the different sides of the zebrafish brain within the mold. R refers to rostral, D for distal, C for caudal, and V for ventral. Once placed in the correct position in the center of the base mold, the rest of the mold was filled with OCT with care not to disrupt the position of the brain sample. It was also essential to ensure there were no air bubbles anywhere near the brain sample within the OCT as this would affect later imaging of the sample. Any bubbles were removed with the corner of a kimwipe or moved with forceps to ensure they were

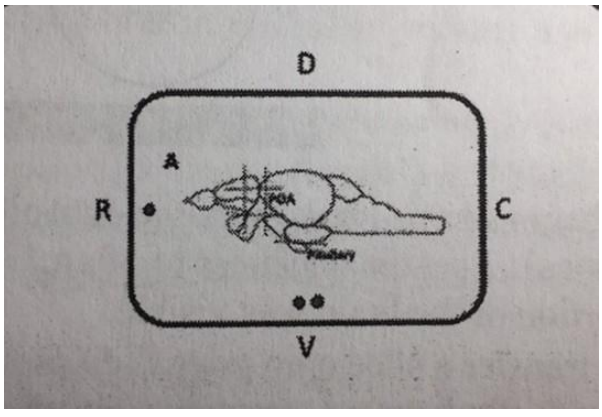

Figure 1: Sagittal view of zebrafish brain within base mold in preparation for freezing

not close to or on top of the tissue. The tissue was then frozen by dipping the mold into liquid nitrogen using long forceps. Care was taken to ensure the only part of the mold coming into contact with the liquid nitrogen was the bottom portion of the mold. The OCT and tissue sample did not come into contact with the liquid nitrogen, but rather were frozen from the bottom up. The tissue was completely frozen when the OCT lost all its transparency and became an opaque white color throughout the whole base mold. The brain samples were then ready for sectioning.

Sectioning and Mounting

Once the tissue samples were frozen, they were ready to be sectioned. The cryostat was used for this part of the experimental procedure. The cryostat was set to -19° C and the tissue samples frozen within the base molds were placed within the cryostat to equilibrate for 30 minutes. The cryostat was set to slice the samples at 30 μm thickness. Following equilibration, the brain samples within the frozen OCT were attached to the cryostat chucks with unfrozen OCT. Markings were placed on the mold to ensure that the orientation of the brain was noted correctly. A blade was then used to cut away at the excess OCT around the sample of the zebrafish brain which was in the center of the OCT mold. This was done to minimize the amount of OCT transferred onto the microscope slides once the sample was sliced. It was essential to not cut too close to the center of the OCT mold as the brain sample could be damaged. Once the trimmed down OCT mold was prepared, it was placed within the cryostat and the slicing began. Once the brain was visible amidst the white of the OCT, section collection began on Superfrost Plus Microscope slides. Each tissue slice was collected on the microscope slide by flattening out the slice with brushes, inverting the microscope slide, and coming into direct contact with the slice. Once in contact with the warm slide, the brain slice would flatten onto it and adhere to the slide. This process was repeated until the whole tissue sample was sliced and there was no more brain visible within the white of the OCT mold. This resulted in 4 brain slices per slide and approximately 10-12 microscope slides depending on the size of the zebrafish brain and when collection begun and ended.

Following sectioning, the microscope slides with attached tissue slices were mounted. This was done with ProLong Gold Antifade Mountant. The slices needed to be mostly dry before adding the mountant. Once the tissue slices were dry, a drop of mountant was added to each tissue slice on the microscope slide. Any bubbles from the mountant were removed using the

corner of a kimwipe in the same process as during freezing with the OCT. Once the bubbles were removed, a glass coverslip was placed on top of the tissue slices. This was done carefully and at an angle so that the tissue slices were not harmed, and no other bubbles were formed which could interfere with the imaging process. Once the coverslips were in place, the slides were stored in 4° C dark environment for 24 hours to allow the ProLong Gold mountant to cure. Following the 24-hour period, the sides of the coverslip were sealed to the microscope slides using clear nail polish. The slides were then ready for the imaging process.

Using the transgenic zebrafish line (*Tg(dat:EGFP)* allowed for direct mounting and imaging following the sectioning process. Without the transgenic zebrafish line, wild type zebrafish would have been used and a rigorous immunostaining process with primary and secondary antibodies would have had to been conducted in order to see the fluorescence of the dopaminergic neurons. This was attempted at the beginning of this study, and resulted in many errors due to the intensity of the process. Many brain samples were lost due to the repeated washing process of the samples during immunostaining. As a result, the transgenic line was used instead of the wild type line which saved much time and materials.

Image Acquisition and Data Analysis

The slices were then imaged using a confocal microscope under a 40X oil lens. Several images were taken of each brain slice depending on what areas of the hypothalamus were visible in that particular brain slice. The areas imaged were the A11, A10, pvopA, and pvopB. Once the images were acquired with the confocal microscope, they were analyzed using the Imaris software. The neurons within the areas of interest were counted by placing a “dot” on each neuron, as can be seen in the example of the pvopB in figure 2. Once all the images were analyzed and all the

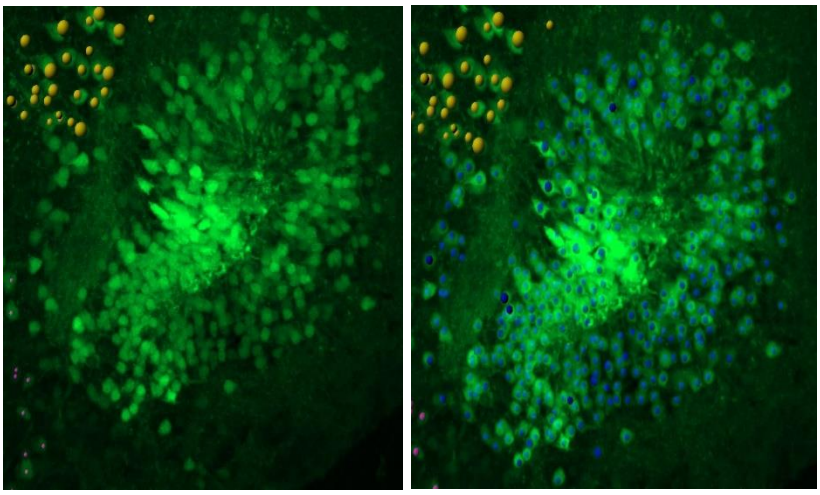

Figure 2: Image of the pvopB in a dominant animal brain slice before (left) and after (right) image analysis with Imaris software. A blue "dot" was placed on each dopaminergic neuron in the area of interest.

dopaminergic neurons were counted with the software, the numbers for each brain region for each animal, dominant or submissive, were tabulated and analyzed using the GraphPad Prism software. The results were shown to be statistically significant with an asterisk over the graphs that had a p-value less than 0.05.

IV. Results and Discussion

Results

There was a sample size of 7 for this study (n=7) of dominant and subordinate fish pairs. The four major areas analyzed were the A11, A10, pvopA, and pvopB.

Analysis of the A11 region was the main focus of this study. While observing the neurons during confocal imaging and data analysis, it was seen that the morphology of these neurons is unique in that they are significantly larger and brighter than all of the other dopaminergic neurons within the hypothalamus. They also have long and obvious axonal projections. These morphological characteristics can be seen below in figure 3. The number of neurons in this hypothalamic region in both dominant and submissive fish ranged from 50-150. It was also observed after analysis that there were more A11 neurons in the dopaminergic animal hypothalamus than the submissive animal. There was a statistically significant difference between the number of dopaminergic neurons in the A11 of the dominant versus submissive fish. This can also be seen in figure 3 and in figure 4.

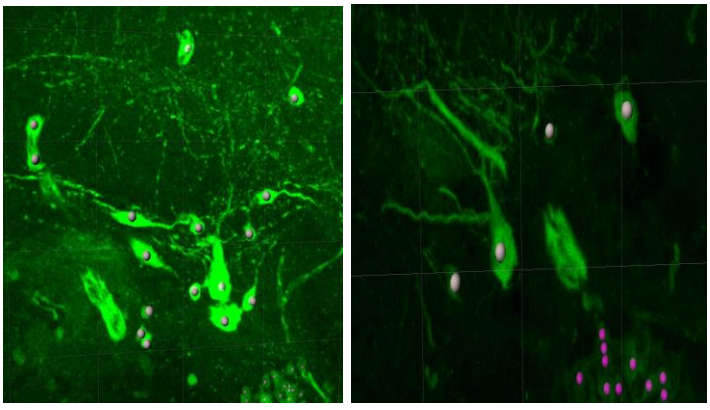

Figure 3: Dominant animal brain slice of the A11 region (left) and submissive animal brain slice of the A11 region (right) following image analysis using the Imaris software placing grey "dots" on neurons.

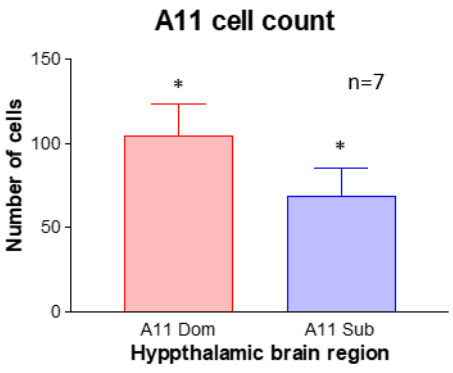

Figure 4: Tabulated data of 7 dominant and submissive zebrafish pairs showing statistical significance in the difference between number of dopaminergic neurons in the A11 region.

The A10 region was also analyzed during this study. The neurons of the A10 were most similar in size and axonal projections to the A11 neurons than any of the other analyzed areas. This can be observed in figure 5 below. The number of neurons in this hypothalamic region, in both the dominant and submissive fish, increased compared to the A11 region and ranged from 400-750. A similar result was obtained in this hypothalamic region that there was a statistically significant difference in the number of neurons in the A10 region between the dominant and submissive fish. This can be observed in figure 5 and figure 6 below.

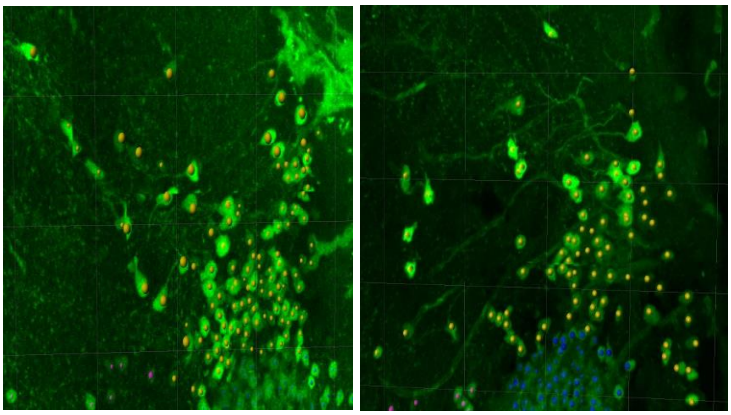

Figure 5: Dominant animal brain slice of the A10 region (left) and submissive animal brain slice of the A10 region (right) following image analysis using the Imaris software placing yellow "dots" on the neurons.

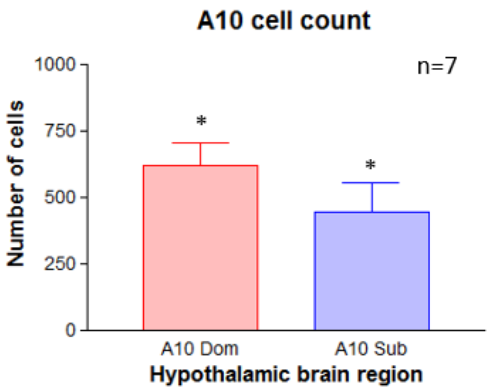

Figure 6: Tabulated data of 7 dominant and submissive zebrafish pairs showing statistical significance in the difference between number of dopaminergic neurons in the A10 region.

The pvopA was also analyzed during this study. These neurons were unique because they had a different morphology than both the A11 and A10 neurons. These neurons were noticeably smaller and did not have any obvious axonal projections. This can be seen in figure 7. There were also many more neurons in this region in both the dominant and submissive animals compared to both the A11 and A10 regions, with numbers ranging from 3000-5000 neurons. Following analysis of the pvopA, it was noted that there was not a statistically significant difference between the number of neurons in this brain region between the dominant and submissive animals. This differs greatly from the previously discussed A11 and A10 regions and can be observed in figures 7 and 8 below.

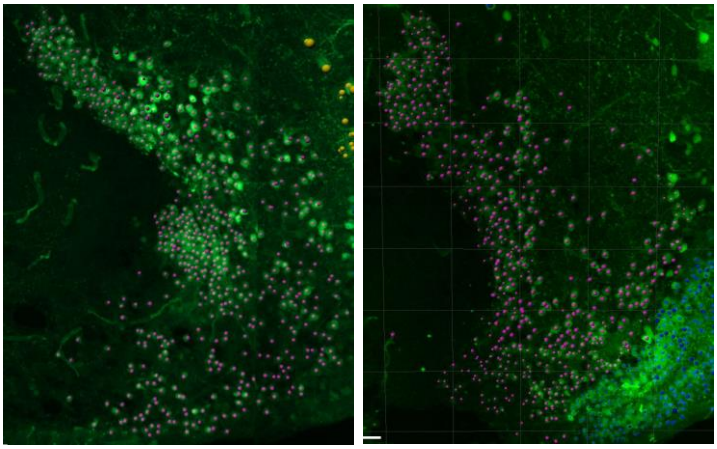

Figure 7: Dominant animal brain slice of the pvopA region (left) and submissive animal brain slice of the pvopA region (right) following image analysis using the Imaris software placing purple "dots" on the neurons.

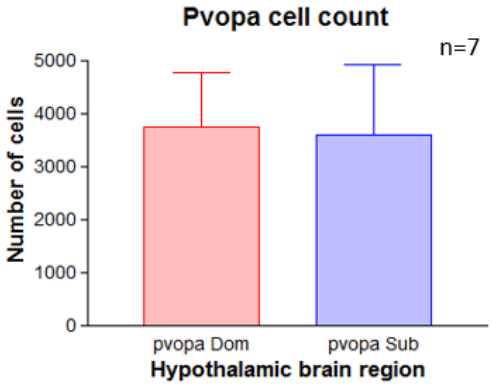

Figure 8: Tabulated data of 7 dominant and submissive zebrafish pairs showing no statistical significance in the difference between number of dopaminergic neurons in the pvopA region.

The final region analyzed in this study was the pvopB region. The neurons in this hypothalamic region were most similar to the neurons of the pvopA region. The morphology of these neurons was also small and clustered with no obvious axonal projections. The pvopB region was essential because it was an identifier to help orient the researchers within the hypothalamus. This region is notable for having the appearance of a 'parsley bundle' and once this region was found, the other regions were easier to locate. The morphology and appearance of the cluster can be seen in figure 9. Also like the pvopA, there were many more neurons in this region compared to the A11 and A10 in both dominant and submissive fish, with number ranging from 4050-7000. This region was also comparable to the pvopA in that there was no statistical significance between the difference in number of dopaminergic neurons between dominant and submissive animals in the pvopB. This is observed in figures 9 and 10 below.

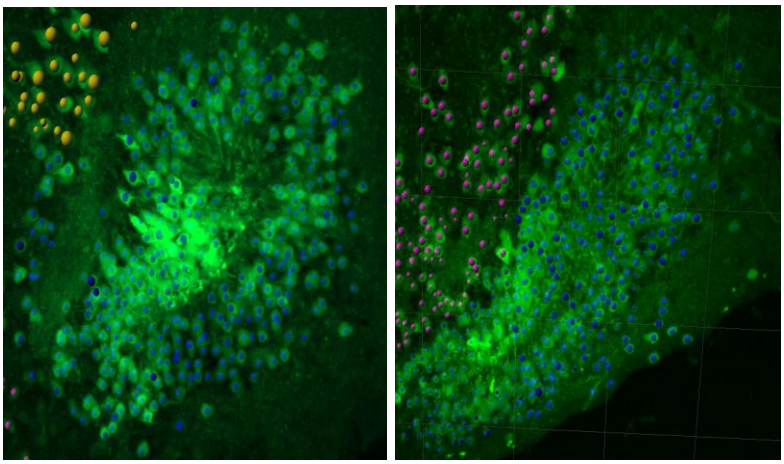

Figure 9: Dominant animal brain slice of the pvopB region (left) and submissive animal brain slice of the pvopB region (right) following image analysis using the Imaris software placing blue "dots" on the neurons.

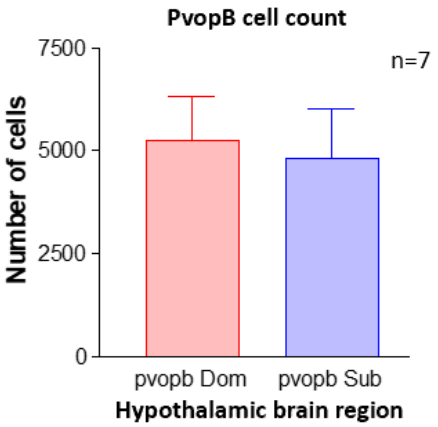

Figure 10: Tabulated data of 7 dominant and submissive zebrafish pairs showing no statistical significance in the difference between number of dopaminergic neurons in the pvopB region.

Discussion

Knowing that the dopaminergic nuclei have a major role in motivation, reward, aggression, and stress and that being socially dominant is a rewarding status, the hypothesis was that socially dominant animals would have a larger number of dopaminergic neurons in the hypothalamus. This result of this study was that the A11 and A10 regions of the hypothalamus had a statistically significant difference in the number of hypothalamic dopaminergic neurons between the dominant and submissive fish, with the dominant animals having more neurons than the submissive animals. However, the pvopA and pvopB regions did not have a statistically significant difference in the number of dopaminergic nuclei between the dominant and submissive fish. The A11 neurons are known to project directly to the spinal cord. This hypothalamic region has the most research regarding the projection to the spinal cord and the Mauthner neuron, therefore allowing these nuclei to influence behavior and locomotion based on social status. The results emphasize this finding because when the animal displayed a dominant social status, the results show that there was also an increase in the dopaminergic nuclei within the A11. This points to morphological changes in the dopaminergic nuclei resulting from the social dominance. In a similar way, when the zebrafish showed social submissiveness, there was a morphological plasticity which resulted in a lower number of dopaminergic nuclei in the A11.

The A10 had a similar result which agrees and further supports the result because it is the only other hypothalamic dopaminergic region studied where the neurons also display projections.

The pvopA and pvopB regions did not display any statistical significance between the number of dopaminergic nuclei in the dominant versus submissive animals. This proved to be an important negative control in the study because it supported that it was social status that was causing the morphological difference in number of neurons between the dominant and submissive animals in the regions with axonal projections, A11 and A10. It is possible that the pvopA and pvopB are not involved in social status regulation like the other regions, and it is postulated that these regions could have more of a role in homeostatic control. This could also help explain why these regions had so many more neurons clustered together than the other regions and why there was so much similarity in their abundance. This is postulated because the hypothalamus is known to play a major role in homeostasis within most vertebrate species, and it is known as the master homeostatic regulator for a wide range of physiologic processes.[3]

In conclusion, there are morphological changes in the A11 and A10 regions of the hypothalamus based on the social status of the fish – dominant or submissive, where dominants have more neurons that their submissive counterparts. Also, there is a negative control with the pvopA and pvopB regions because they do not show a difference in neuronal number based on the social status of the zebrafish. The results demonstrate that social dominance induces morphological reconfiguration of the hypothalamic dopaminergic system in a social status-dependent manner. Some of the broader impact of this study is that social status can selectively influence the morphology of the hypothalamic dopaminergic neurons underlying motor behavior. This could play a role in the aggression displayed by other social animals and our results are likely to have broader implications on other vertebrate social species in future studies.

V. References

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