LOCALIZATION OF NEURAL CIRCUITS INVOLVED WITH ENCODING, STORAGE, AND RETRIEVAL OF SPATIAL MEMORIES IN THE TELEOST BRAIN

By

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A thesis submitted to the

Graduate School-Camden

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of Master of Science

Graduate Program in Biology

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Camden, New Jersey

October 2018

ABSTRACT OF THESIS

Localization of Neural Circuits Involved with Encoding, Storage, and Retrieval of Spatial Memories in the *Teleost* Brain

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Declarative memories are paramount for the survival of an individual and their species. Declarative memories are those which are formed and reinforced through the personal history and experiences of an individual. The mammalian hippocampus plays an irreplaceable role in the formation of declarative and spatial memory. The hippocampus is vital in forming and storing a new memory, yet not completely necessary for the recall of a previously established memory.

Teleost fish possess the capacity to form declarative memories, however, the homologue to the mammalian hippocampus in the teleost brain is not clearly defined. Due to differences in embryonic development of the prosencephalon (invagination vs eversion), topological comparisons of the mammalian and teleost brain cannot directly identify corresponding regions.

To locate the areas in the teleost brain equivalent to the hippocampus, we devised an experiment that focuses on cell activity during memory formation. The telencephalon regions most active in the teleost brain during spatial memory formation are located and compared to the same areas during memory recall. The regional differences in activity

between forming a memory and recalling a memory in a teleost may identify the equivalent to the hippocampus. The study focuses on three regions of the dorsal pallium of the teleost forebrain selected as probable regions of interest based on previous studies and literature. The study uses cytochrome oxidase activity as a measure of the most recent cellular activity in the investigated regions.

A naïve group was used as a control. An experimental group was trained to learn a spatial oriented task. A recall group, taught the same task as the experimental group, but left inactive for 2 weeks, was forced to recall the same task. After the forced recall, their forebrains activity was immediately studied.

The experimental group displayed a significant increase in activity in Dm compared to the recall group. The difference in activity between experimental and recall groups show activation of Dm during learning the spatial tasks, not during recall of the learned tasks. Dm appears to be part of the telencephalon used for encoding and storing memories, not the recall of the same learned task.

ACKNOLEDGEMENTS

I would like to thank my P.I. Dr. William Saidel for all his assistance, patience, and insight on this project. I also would like to thank my lab mate Christina Curran-Alfaro for all the time and effort she brought to this project. Thank you to my committee members Dr. Amy Savage and Dr. Joseph Martin for their help and input along the way. Thank you to Jigar Patel for writing the Image J plugin which allowed me to more efficiently acquire data. Thank you to Dr. Alejandro Vagelli for his knowledge in marine tagging. Thank you to many others who helped along the way as well.

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INTRODUCTION

Memory plays a vital role in survival and success in most living species. An important aspect of survival is to know where one is. Spatial memory formation in mammals has been shown to be heavily dependent on the region of the brain known as the hippocampus (Fig. 1) (Deadwyler, 1980). The mammalian hippocampus is an area of allocortex located along the medial side of the temporal lobe (Squire, 2008) and is connected to adjacent areas such as the parahippocampal, perirhinal, and entorhinal cortices (Zolamorgan and Squire, 1993). When a memory is formed, the first step is the sensory cortex processing all stimuli involved. During this processing the brain determines what stimuli and information will be retained or discarded in association with the established memory. The second step is the actual storing of the memory for recall. Both events are necessary components for formation of a memory. Subsequent to the initial sensory processing, the relevant step for memory formation takes place in the hippocampus (McGaugh, 2000). The storage of a memory may occur elsewhere. Different areas of the brain may be associated with one or more roles in memory formation and recall. In mammals, the hippocampus is the necessary tool for establishing a class of memories called declarative memories (Hannula and Helmstetter, 2016).

Declarative memories are memories of personal history and experience. These memories are of undeniable importance for the survival of an individual. Survival of individuals equate to survival of the species (Kolarik et al., 2018). In mammals, memory

formation occurs as an integration of multiple stimuli consequent of a specific event at a specific period in time (McGaugh, 2000). A result of the convergence and integration of the stimuli, cells in specific regions of the brain are permanently altered (Alme et al., 2014). The combination of permanent changes in the locations involved with this integration can be called a memory trace, or "engram" (Choi et al., 2018). This engram is then a specific declarative memory and, these engrams are central to navigational memories (Kolarik et al., 2018).

Fish possess the ability to form a declarative memory (Rodriguez et al., 2002). Individual fish species around coral reefs must be able to return to their own microhabitat within a reef after spending time in other areas of the reef foraging (Noda et al.,1994). The teleost brain is able to form such declarative memories for survival of its species. Reef fish are able to navigate very large, dense, and complex territories without losing their point of origin (Noda et al.,1994). The reefs that these fish navigate contain multiple types of sensory stimuli along with many potential locations to be remembered. In order for a memory to be formed, all the sensory information being gathered at that moment, (sight, sound, temperature, smell, feeling, time, proprioception, etc.) must first be received and processed by specific areas in the telencephalon. During processing unnecessary information may be filtered and omitted from the actual consolidation of the memory (Turatto et al., 2018). After the initial processing, the information and stimuli must be sent to a network containing place cells for encoding (Squire, 2008).

The homologue to the mammalian hippocampus in the teleost forebrain is undefined. The problem in identifying the hippocampal region in the teleost brain, is that one cannot use traditional comparative neuroanatomy due to the differences in embryonic

brain development between mammals and ray-finned fish (Butler, 2011). In the early stages of embryonic development, the anterior end of the neural tube of both mammals and ray finned fish develops into a brain (Fig 2).

The area investigated in this study develops from the most anterior vesicle of the neural tube to become the teleost forebrain or prosencephalon (Ebbesson, 1980). The prosencephalon of a mammal develops into a telencephalon through a process of invagination. While the invagination process occurs, regions of the brain undergo cellular hypertrophy to develop into the mature telencephalon. In contrast to the mammalian brain, the prosencephalon in teleost develops in a different manner. In an embryonic teleost, the development of the anterior vesicle forms a telencephalon by an eversion of the neural tube, also due to cellular hypertrophy (Ebbesson, 1980). The areas in the mammalian brain that usually bend inward from the hypertrophy turn outward (M. R. Braford, Jr. and Northcutt, 1974). This makes comparative topographical anatomical comparison of the brain regions difficult.

Relative comparisons can be made of brain regions developing from corresponding neural tube locations between mammal and teleost (Fig. 2). However, it would only be an assumption to state that these areas perform the same function based on their corresponding original locations before cell proliferation (Butler and Saidel, 2000). Comparative geographical neuroanatomy may be used as a starting point, but other means to locate the hippocampal homologue in the teleost brain must be used. To better understand the homologue to the mammalian hippocampus in teleost brains, neural connections most heavily utilized during the spatial learning process were studied (Leutgeb et al., 2005).

Memories are incorporated into working memory and long-term storage. Recall can draw from both working memory and long-term storage (McGaugh, 2000). The hippocampal cells in mammals link engrams with specific places (Alme et al., 2014). These cells, linking a memory to a place, are known as place cells. These place cells and their associated locations in the teleost forebrain, (Ocaña et al., 2017) are the target areas studied in this experiment in an effort to locate the hippocampal homologue in the teleost brain.

In this study the test subjects (*Carassius auratus*) learn a spatial oriented task through behavioral training. The task is intended to induce a memory of a specific spatial location in a three-dimensional tank. The fish must navigate through the behavioral training tank to locate a specific area to complete the task. Once the subjects achieved criterion of having learned and repeated the required spatial learning task they were sacrificed. The areas of the forebrain under investigation in this experiment were processed for cytochrome oxidase activity. Cytochrome oxidase activity identifies the cells in regions of the brain with both a most recent increase in ATP use and long term usage (Wong-Riley, 1989). The increase in ATP use is indicative of cellular activity in the memory formation (engram) and in recall tasks. The trained subjects are compared against a naïve cohort, and against a cohort trained and tasked to recall the same behavioral task 2 weeks after training.

The hippocampus and “engram” may be two separate and equally important parts in formation of a memory. The hippocampus is important in processing the memory, and the “engram” is important in storing the memory for later recall (Leutgeb et al., 2005). One need only look at the case of H.M. to see that memory recall or engram sustainability

is possible without hippocampal activity. Patient H.M., a famous neurological case, was able to recall declarative memories from the portion of his life before the near complete removal of the hippocampal areas of his brain (Neylan, 2000). When asked to recollect declarative memories from the time period after his operation, patient H.M. suffered from a severe case anterograde amnesia (Neylan, 2000). The memories processed by his pre-surgery hippocampus were available for recall in the absence of his hippocampus (Neylan, 2000). His brain had lost its processing or encoding abilities for new memories, not the pre surgery memories or recall capabilities (Neylan, 2000).

The behavioral training in this study is designed to stimulate the same type of spatial episodic memory formation which involves the hippocampus in mammals. By comparing areas in the teleost telencephalon activated during behavioral training, memory formation, and memory recall tasks, we may be able to find the hippocampal homologue. The recall cohort in this study is an additional variable which investigated the activity in the areas of the teleost forebrain during memory recall. The differences in forebrain activity across treatments depict shed light on hippocampus vs engram in the teleost brain.

Methods and Materials

Over the course of this study, multiple cohorts of goldfish (*Carassius auratus*) were obtained from a local fish supplier. All test subjects were housed in the same 30-gallon freshwater tank under controlled conditions. After approximately 48-72 hours of acclimation to the tank, test subjects were tagged for identification purposes.

Tagging:

Test subjects were implanted with a "Visible Implant Elastomer" tag manufactured by Northwest Marine Technology Inc (Fig.3). The Visible Implant Elastomer tags allowed for the individual identification of each subject while being housed in a communal tank. (The importance of the communal tank cannot be underestimated. It provided for a common experience prior to any experimentation.) The V.I.E. tags were injected below the surface of the skin as a liquid where it dried into a pliable solid. A total of 6 different locations were chosen along the superior sagittal midline of the test subjects body (Fig.4). The locations chosen were, right rostral, left rostral, right dorsal, left dorsal, right caudal, and left caudal. With the combination of two colors, 6 locations, and up to 4 tags per a fish, a possible total of 240 subjects could be individually identified. The test subjects were allowed another 72 hours of inactivity in the communal control tank before any behavioral training began. After the 72-hour inactivity period the subjects were evaluated for tag retention. After verifying tag retention and individual identification of all subjects, behavioral trails commenced.

Experimental Environment:

The experimental tank was fabricated in the Neuromorphometry lab at Rutgers-Camden from clear acrylic plexiglas. The dimensions of the tank measure 84 inches x 1.5 inches x 5 inches (Fig. 5). The clear tank was then wrapped in a vinyl sheath faced with an arbitrary black and white pattern. For purposes of the experiment, 3 inches of the pattern on the vinyl wrap was colored with red, blue, and orange water-soluble ink. The colored area indicated to fish a "feeding zone." In the bottom of the tank centered in the feeding zone is a photo sensor. When a fish entered the feeding zone and activated the photo sensor, a small amount of fish flake food was automatically dispensed into the water above the feeding zone. Simultaneously, a red, light emitting diode was activated on the right side of the feeding zone.

Behavioral training time was defined as the time between entry of the tank to reaching the feeding zone. The first cohort of 15 fish was used to set a criterion or standard of learning, along with a learning curve. It was determined that after a series of 5 trials in the experimental tank, that criterion was achieved. The standard was set when 12 of 15 subjects had reduced their time to the feeding zone by 80 percent or better (Fig. 6).

Obtaining Experimental Material:

After criterion was reached by a new cohort, these subjects were anesthetized with approximately 0.1 grams of ethyl-3-aminobenzoate methanesulfonate salt in 100 ml of aquarium water (0.1%). After tail reflex test elicited no response and respiration ceased, subjects were prepared for profusion by bilateral removal of operculum and pectoral fins. The heart was located and exposed. The atrium of the heart was cut to relieve pressure and to allow saline solution to flow more freely into the vascular system. A 22-gauge needle was inserted into the conus medullaris of the heart and 0.7 percent

saline solution was injected into the subject's system. Saline was pushed through the subject's system until exsanguination was complete. A combination 2% paraformaldehyde, 0.5% glutaraldehyde fixative was administered via the same delivery system as the saline solution. Once fixed, the subject's brain was removed and immersed in the same fixative solution for 1 hour. The brain was placed into thirty percent sucrose buffer until it sank, after which, it was surrounded with twelve percent gelatin solution and placed in refrigeration until the gelatin solidified. The block was fixed in the same fixative only containing 30% sucrose, and trimmed down to a workable size. The final block was notched on one side to use a point of reference when later determining laterally of sectioned images. After preparation of a brain into a gelatin block was complete, it were frozen sectioned at 40 microns on a sliding microtome. The forebrain was oriented superiorly, and transverse serial sections were made. Each subsequent sequential section was placed in a well containing buffer solution to maintain serial order.

After completing sectioning, the histochemistry staining was performed. Sections were placed in a solution of 0.1M phosphate buffer, 4 grams of sucrose, 20 milligrams cytochrome C, and 60 milligrams diaminobenzidine. The brain sections in the solution were then placed in an oven at 37^{o} Celsius. The sections were held at 37^{o} Celsius for approximately 3 to 4 hours until a visible darkening of nervous tissue occurred. Once stained, the sections were washed 3 times in 0.1M phosphate buffer (Wong-Riley and Welt 1980). Each individual brain section beginning with the most rostral forebrain section was mounted on a glass slide. Serial integrity of the brain sections was maintained by mounting in this manner. Slides were dehydrated and cover-slipped. Each section was photographed at 5x under Kohler illumination using a Zeiss Axioplan

microscope with a Nikon D90 digital camera at 4,288 (wide) x 2,848 pixels (tall) per image.

The sections were photographed in the same order in which they were sectioned and mounted. The sequence began with the most rostral section of forebrain followed by the next subsequent sequential section. Each photo was saved as a .tif file to ensure no loss of data. Each photo was saved as its own file and given a label which indicated which particular fish, slide, row and section.

Processing:

Each individual photo was imported into ImageJ using the same serial identification denoting the origin of the photo. Each photo was separated into its respective red, green, and blue channels, each channel denoted by a 1 to 256 scale LUT. I determined that the green channel provided the best quality contrast of the images. The red and blue channels were subsequently discarded. The images of the green channel were then converted to grayscale and all set to a LUT standard 1 to 256 LUT scale to ensure uniformity during pixel density analysis.

Areas of Measurement:

Regions of the images were measured using an ImageJ plug-in developed by Jigar Patel. With this plugin, the sections were evaluated based on an algorithm that measures the pixel density in a specific defined area. The pixel density analysis was focused on specific areas in the forebrain identified from literature as likely places to investigate

(Broglio et al., 2010; Ocaña et al., 2017; Rodriguez et al., 2002; Portavella et al., 2004; Braford, M. R., 2009; Saito, K., & Watanabe, 2006; Uceda et al., 2015).

Pixel density measurements of the relevant areas from the telencephalon of trained subjects (*the experimental group*) were compared to the same areas from naïve subjects (*the control group*) and from subjects who had been trained, left inactive for 2 weeks before being asked to repeat the task (*the recall group*). The comparison of the values taken from the control group against the values taken from the fish of the experimental group provide the data for possible location(s) of the "hippocampus" and storage loci. The comparison of the values taken from the forebrains of the control group against the values taken from the recall group provides the data for possible locations for memory storage. The difference in comparison of the data from the experimental group with the date from the recall group of fish reveals a targeted area for the teleost homologue to the mammalian hippocampus.

From previous studies the primary telencephalic regions being investigated are the area dorsalis telencephali (D), to include Central zone (Dc), the Dorsal zone (Dd), the lateral zone (Dl), the dorsal part of lateral zone (Dl-d), the Ventral part of lateral zone (Dl-v), and the Medial zone (Dm) (Fig.8) (Braford, M. R., 2009; Broglio et al., 2010; Ocaña et al., 2017; Portavella et al., 2004; Rodriguez et al., 2002; Saito, K., & Watanabe, 2006; Uceda et al., 2015).

Image analysis

The areas in question were measured using an ImageJ plugin. Four loci from each region were sampled to produce 4 different pixel density values. each value was the average of all pixel values from within a circle. Each circle was set to a standard area of 1257 pixels or 706.86 microns^2 (Fig. 9). The values were entered into an excel spreadsheet and pixel density averages and standard deviations were calculated for each brain region with respect to all subjects within each treatments. The values of each treatment were compared against the others. Since the pixel density scale is a range of values from 1 to 256, 1 is the darkest black on the scale and 256 is the brightest white. Thus, lower value for a pixel density measurement represents a higher level of a COX activity.

Statistical Analysis

Assumptions of the General Linear Model were performed in R Studio. A Shapiro-Wilks test was preformed to ensure normality of the data. The homogeneity of the data was also tested in R Studio using a Bartlett test. After concluding GLM was not violated, the data was analyzed in Origin Pro 2018b. The analyses performed included a Sphericity test, a test of the factors among and within subjects, and a test among the sample sizes. All were conducted as part of a two-way repeated measures ANAOVA analysis.

Results:

Behavioral Training Results:

The initial cohort of 15 fish was taught the self-feeding task in the trial tank to establish a 'learning curve'. The criterion for reaching success at the task was set at an 80% decrease in initial time to the feeding zone (Fig. 6). Graphs and the normalized times of all the subjects of the first cohort were made.

Figure 7 is the graph for the first recall cohort in which trials 1-4 were trained in succession. Once trials 1-4 were complete and criterion achieved, the test subjects were returned to the holding tank. There, they were housed and fed regularly for a period of 14 days without training or retesting. After 14 days, the recall cohort were reintroduced to the trial tank. The majority of the recall cohort showed a further decrease in time on the fifth trial. After 1 day of inactivity, on the sixth trial, the recall cohort showed a further decrease in time compared to their initial trials 1-4.

The increasingly successful reduction of time to feeder in trials 5 and 6 of the recall cohort suggests a successful storage of the engram associated with the spatial location and function of the feeder and feeding zone. The further reduction of time also suggests that the recall group was able to actively recall the memory of the purpose of the trial tank (feeding), the specific location of the feeding zone in the trial tank, and how to trigger the feeder in the feeding zone of the trial tank. After 14 days of inactivity, the test subjects were able to recall these memories with no visible signs of memory loss. This suggests that the recall of these memories can occur for some period of time well after behavioral training has ceased.

Cytochemistry results

No significant statistical differences in cytochrome oxidase activity were observed for reciprocal loci across the hemispheres. Therefore, numerical measures for the 3 areas of investigation were averaged from both hemispheres into their respectively named region. Three regions, Dm, Dld, and Dlv, were further scrutinized against each other with respect to the treatments. Measurements for naïve, experimental, and recall cohorts were averaged separately. The calculated pixel density value average for the naïve or control cohort was established at 112.13 ±2.25(S.E.M). The pixel density measurement for the recall group was calculated at 105.45±2.96, and the same calculations assigned the Experimental cohort with an overall pixel density value of 97.65 ±3.03. With the naïve cohort as the baseline control group, the recall cohort displays an overall 5.95% increase in COX activity in the overall average of the investigated regions. When examining the experimental group against the baseline naïve cohort, an increase of 12.91% is observed in COX activity (Fig. 10; Table 1). A two-way repeated measures ANOVA was preformed using OriginPro 2018b, and comparisons between regions and treatments displayed significant p values for the areas of Dlv-N vs Dlv-X ($p = 0.000506$), Dlv-N vs Dlv-R ($p = 0.002480$) Dm-N vs Dm-R ($p = 0.04776$), and Dm-X vs Dm-R ($p = 0.000153$) (Table 2).

Experimental vs Naïve

When compared with the naïve treatment, the experimental cohort displays overall increases in cox activity across all regions (Fig. 11). Dld region of the forebrain in the experimental cohort displayed an 8.49% increase in COX activity when compared to the naïve control cohort. Dlv region of the forebrain in the experimental cohort displayed a significant 17.18 % increase in COX activity when compared to the naïve control cohort. Dm of the forebrain in the experimental cohort displayed an increase of 12.63% in COX activity when compared to the naïve control cohort.

Recall vs Naïve

When compared with the naïve treatment, the recall cohort displays a significant increase in the Dlv (Fig. 12). Dld of the forebrain in the recall cohort displayed a non-significant increase of 3.67% in COX activity when compared to the naïve control cohort. Dlv of the forebrain in the recall cohort displayed a significant 15.67% increase in COX activity when compared to the naïve control cohort. Dm of the forebrain in the recall cohort displayed a non-significant decrease of 2.23% in COX activity when compared with the naïve control cohort.

Experimental vs Recall

When compared with the recall treatment, the experimental cohort displayed a significant increase in COX activity in only the Dm of the forebrain (Fig. 13). The Dld in the forebrain of the experimental cohort displayed a non-significant 5% increase in COX activity when compared to the recall cohort. Dlv of the forebrain in the experimental group displayed a non-significant 1.79% increase in COX activity when compared to the recall

cohort. Dm of the forebrain in the experimental group displayed a significant 14.53% increase in COX activity when compared with the recall cohort.

Statistical Analysis

Stemming from the results of a two-way ANOVA repeated measures test of treatment vs region, the Pillali's Trace, Wilks' Lambda, Hotelling's, and Roy's Largest Root tests all produces P values of less than 0.05 (Table 3). All P values for tests of within-subjects effects (Sphereicity assumed, Greenhouse-Geisser, Huynh-Feldt, and Lower-Bound) were below the threshold of 0.05 (Table 4).

Discussion

The intent of this study was to further investigate areas in the teleost forebrain associated with spatial learning, memory formation, and memory recall. The specific areas investigated were proposed by earlier studies to be a possible location for the homologue of the mammalian hippocampus (Butler and Saidel, 2000; Hannula and Helmstetter, 2016; Broglio et al., 2010; Ocaña et al., 2017; Rodriguez et al., 2002; Portavella et al., 2004; Braford, M. R., 2009; Saito, K., & Watanabe, 2006; Uceda et al., 2015). This study investigated the locations of the forebrain at or caudal to the anterior commissure, mainly, Dld, Dlv, and Dm. The results of the behavioral training followed by cytochrome oxidase staining revealed that all loci in the experimental group (Dld, Dlv, and Dm,) showed higher metabolic activity when compared to the control, the same did not hold true for the recall group. This result suggests that areas used in the initial spatial memory formation, or the "processing" of the memory, are not inherently the same as used in recall.

While the experimental cohort displayed significant increases in metabolic activity when compared to the naïve cohort in Dlv and Dld, the recall cohort compared to naive showed a significant metabolic difference in only one region of the forebrain, Dlv. The recall cohort also displayed similar metabolic activity to the experimental group in Dlv and Dld of the dorsal pallium. The only region between the experimental and recall cohorts that showed statistically significant different metabolic activity was Dm.

Experimental Dld, Dlv, and Dm

Earlier studies suggest that Dlv is associated with memory formation and spatial learning in the teleost brain (Butler, 2000). Some even propose that Dlv working in tandem with Dld constitute the teleost homologue of the mammalian hippocampus (Northcutt, 2006). This study is in agreement with the findings published by Ocaña et al. (2017) that the Dlv is activated with spatial training and states that Dld is not activated. The findings in this study concours that while Dld seems to show activation during memory formation, the level of activation is not statistically significant as the p value for both the experimental and recall treatments when compared against naïve are above that of a 0.05 (Table 2). The increase in COX activity in the two other regions of Dlv and Dm of the experimental group are indicative of being activated during the spatial learning tasks. Dlv in the experimental group displayed largest increase in COX activity compared to the naïve control group. Both Dlv and Dm regions displayed increases in COX activity in the experimental group as well. This increase in COX across these two regions of the dorsal pallium indicates that Dlv and Dm are both active as a part of or whole of the neural network involved with spatial learning and memory formation (Table 2).

The Recall Cohort

The recall and experimental cohort displayed similar COX activity in Dlv relative to the naïve group. This significant increase in COX activity across both treatments in Dlv indicates that it is active in both spatial learning and memory recall tasks. The key difference between the recall and experimental groups is the magnititude of COX activity in Dm. In the recall group we see significantly less COX activity in Dm when compared to the experimental group. COX activity in Dm of the recall group is more similar to the naïve recall cohort than it is to the experimental cohort suggesting a basal level of activity during the process of memory recall.

Dm

The difference in the activity levels in Dm between the experimental cohort and the recall cohort indicates Dm is an active part of the neural network required for spatial learning and memory formation. Dm is not necessary for the recall of the same spatial task-oriented memory. Northcutt, (2006) proposed that Dm of a teleost is functionally homologous to the amygdala of tetrapod. The results from this study do not contradict the proposition that Dm in teleost correspond to the mammalian amygdala. In mammals, the amygdala is known to play a crucial role in memory formation and storage associated with emotional events. Some research indicates that the amygdala is active in processing sensory input during "fear conditioning" (Portavella et al., 2004). This may coincide with an initial fear induced in the trial subjects by the sudden introduction to the trial tank. However, the same initial fear might be expected to fade by the time the experimental group had reached criterion. We see a lack of activity in Dm with the recall group that would be present if the test subjects were simply reacting to fear of the trial tank. While this study does not disagree

with Northcutt (2006), it also does not completely agree either. It may be the case that the Dm in teleost brain contains elements similar to the amygdala while also containing elements similar to the hippocampus as well.

Conclusion:

When considering the results based on cytochrome oxidase activity, this study agrees with prior papers that Dlv of the teleost dorsal pallium is significantly activated during the formation of spatial episodic memories (Northcutt, 2006; Ocaña et al., 2017; Uceda et al., 2015). The same Dlv region is also significantly activated during the recall task of the memory it was involved in forming. This activation of Dlv is not in keeping with the role of the hippocampus being used in only processing memories and not in the role of recalling the memories stored.

The activation of Dm in the experimental group during the learning and memory formation is similar to the statistically significant to the levels seen in Dlv. This shows both regions in the teleost forebrain are used for the initial formation and storage of a memory. In the recall group we see the same statistically significant activation of Dlv. This observation shows that Dlv is active in recalling stored memories.

The low levels of activity of Dm in the recall group is of note here. Results from the cytochrome oxidase show a significant difference in the activity levels of Dm between the experimental and the recall groups. The increased activity of Dm in the experimental group compared to the recall group indicate that Dm is necessary for the process of memory formation and storage. This agrees with findings that lesions in Dm cause spatial learning

deficits (Saito and Watanabe, 2006). The lack of activity in Dm in the recall group indicate that Dm is not involved with the recall or retrieval of the same memory.

Through use of spatial learning behavior trials and cytochrome oxidase this study has investigated areas in the teleost forebrain thought to be homologous to the mammalian hippocampus. By comparing concentration densities of cytochrome oxidase activity present during the behavior trials and comparing them against the differing treatments, this paper proposes that Dm region adjacent to the anterior commissure of the telencephalon in teleost fish is the region homologous to the mammalian hippocampus.

An alternative hypothesis can be argued when interpreting the data acquired from the study. It may be possible that a teleost does not possess an area in the brain homologous to the mammalian hippocampus. The formation of a memory may be a more primitive procedure. A procedure in which the formation and storage of a memory are involved with a more emotional process such as “fear induced” learning (Portavella et al., 2004). This may explain why Dm in the teleost prosencephalon, an area thought homologous to the mammalian amygdala (M. R. Braford, 2009), is activated and necessary during the initial “fear based” memory formation, (Saito and Watanabe, 2006), but not activated during the recall of the same memory.

Figure 1. After, (Bergland, 2015) Sagittal and coronal view of the mammalian (human) hippocampal regions of the brain. (RED)

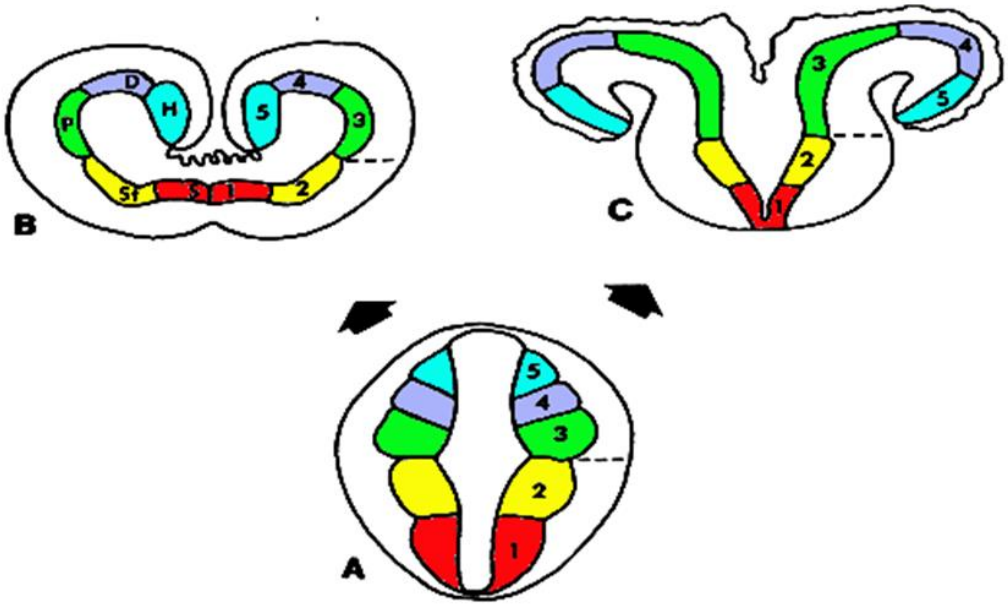

Figure 2. After, (Ebbesson, 1980) A: Representation of neural tube before differentiation to invagination or eversion; B Neural tube after differentiation by invagination (mammalian); C: Neural tube after differentiation by eversion (teleost).

Figure 3. *Carassus auratus* test subject tagged with Visible Implant Elastomer tag in one of 6 loci (right rostral).

Figure 4. Image depicting all 6 possible tag locations.

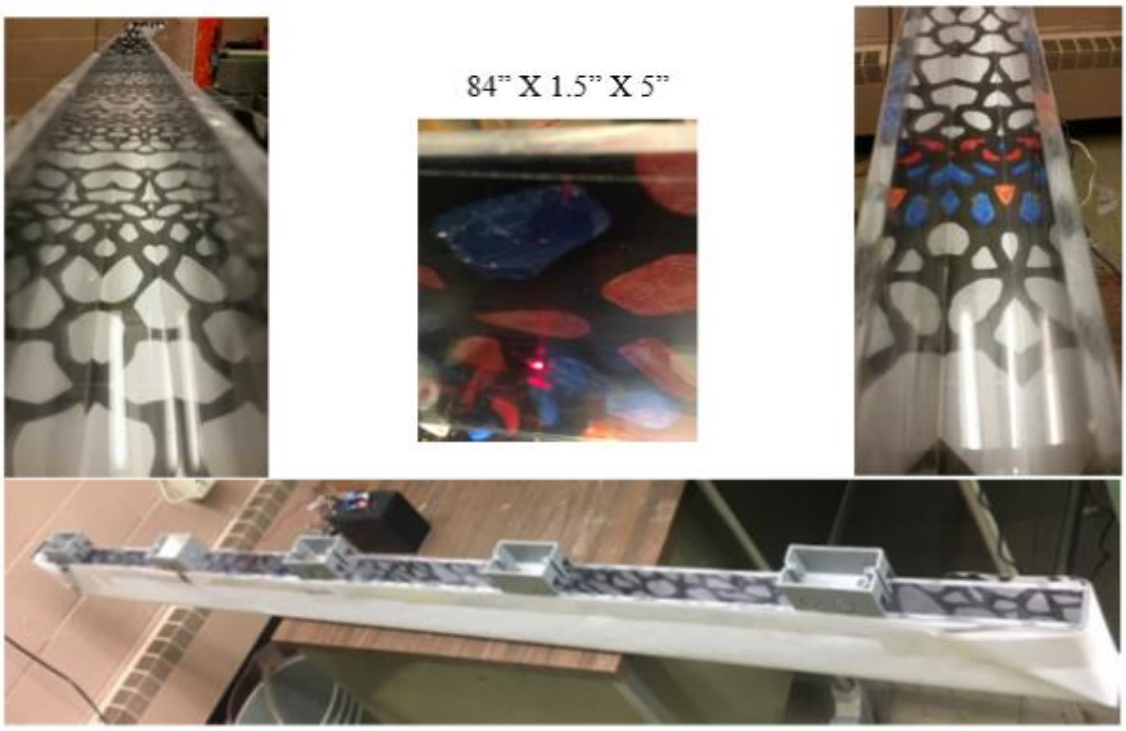

Figure 5. Trial tank used for behavioral training and time trials.

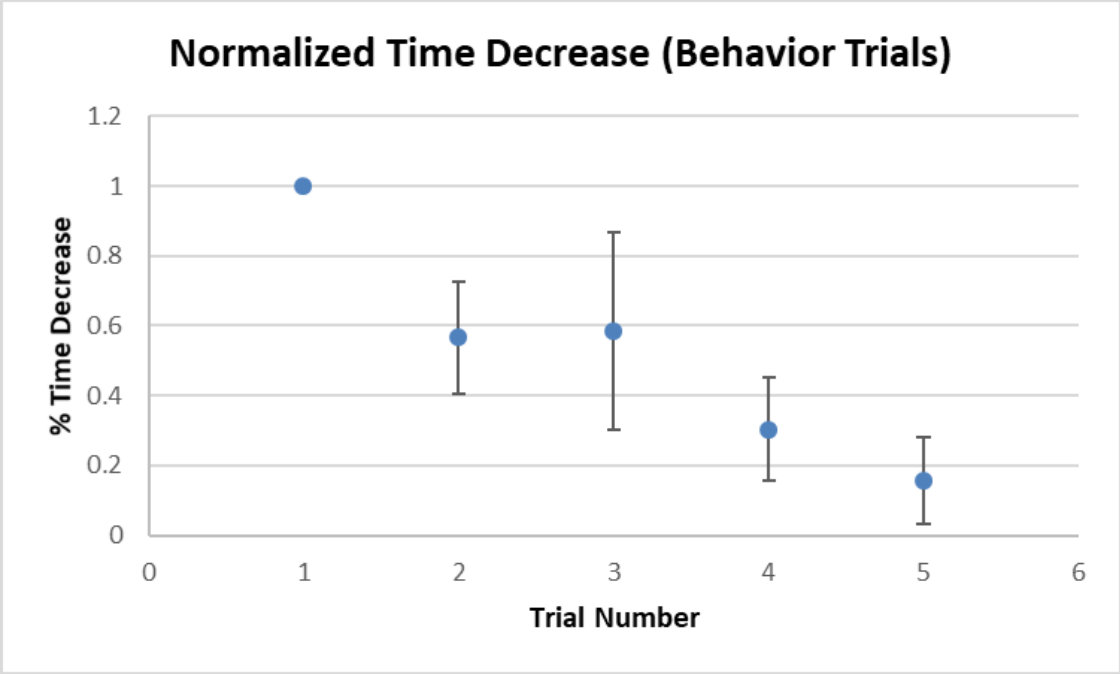

Figure 6. Normalized time averages fish involved in establishing criterion. Trial 5 displays a more than 80%reduction in time.

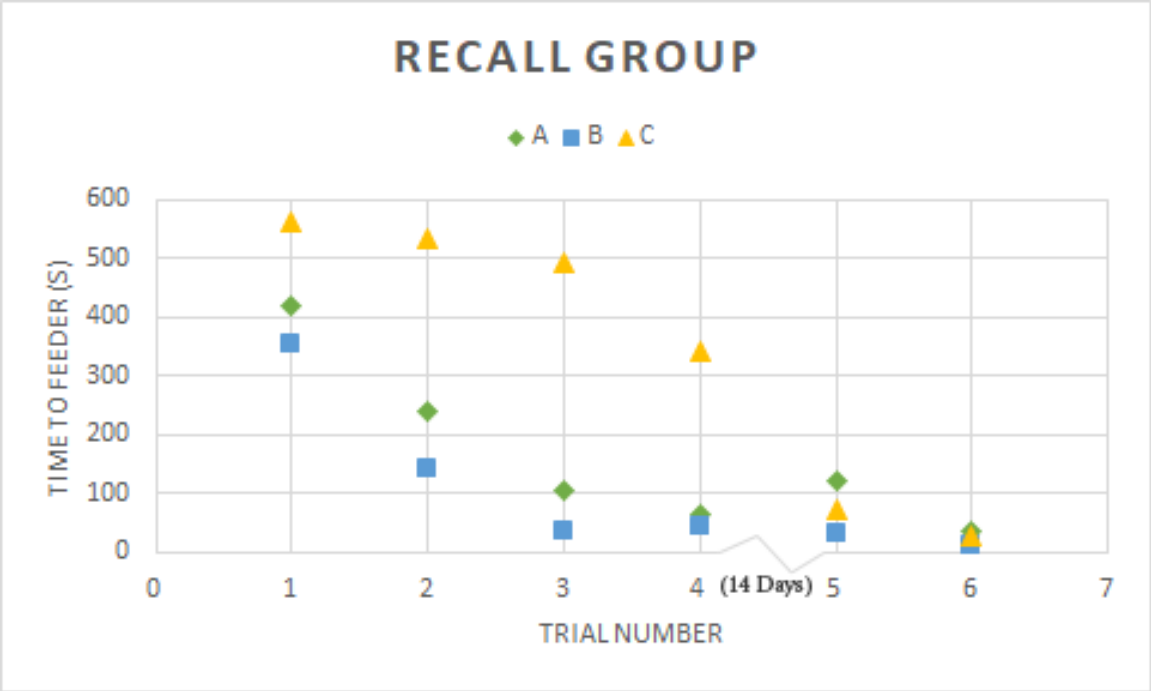

Figure 7: Time to feeder for members A, B, and C of Recall cohort. Note: 2 week time period between trial 4 and 5.

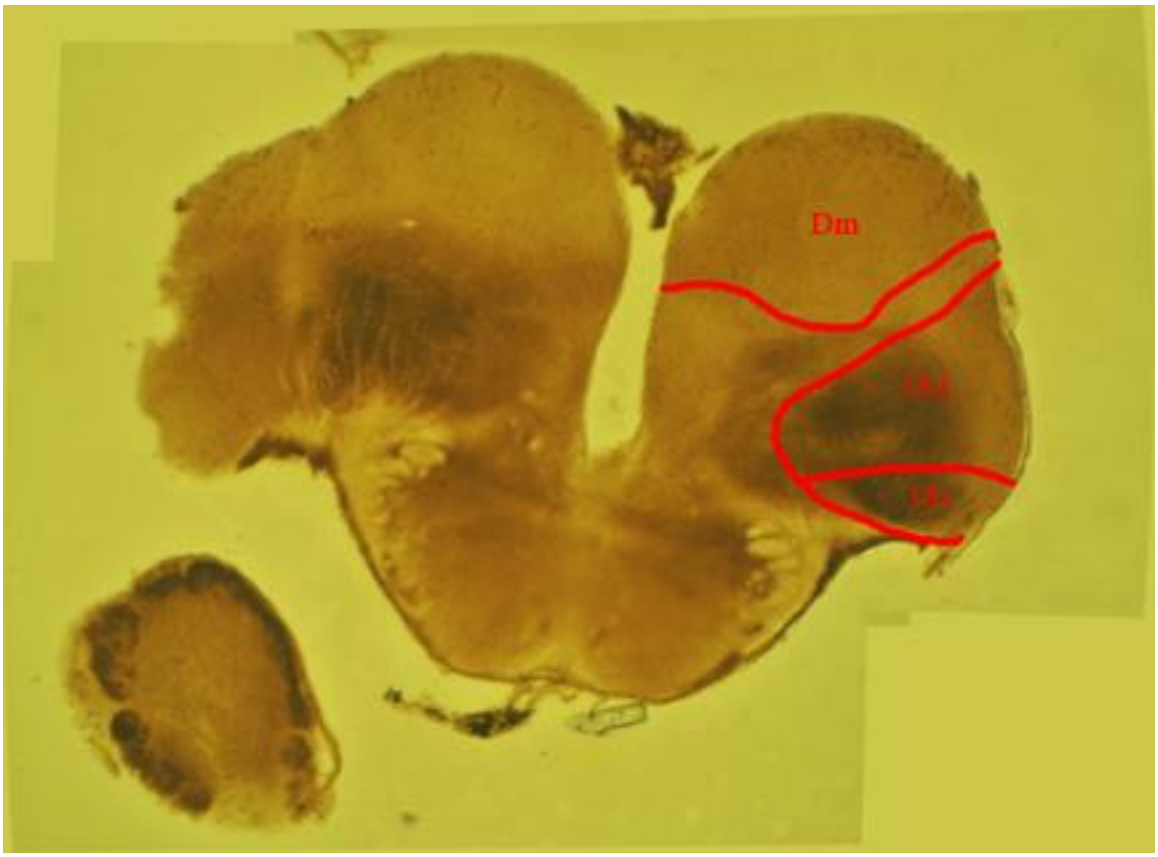

Figure 8. COX stained forebrain with Dm, Dld, and Dlv (X2-1C5)

Figure 9. Top is the Original COX image. Bottom is same image after conversion to greyscale and pixel density analysis with 1mm scale insert.

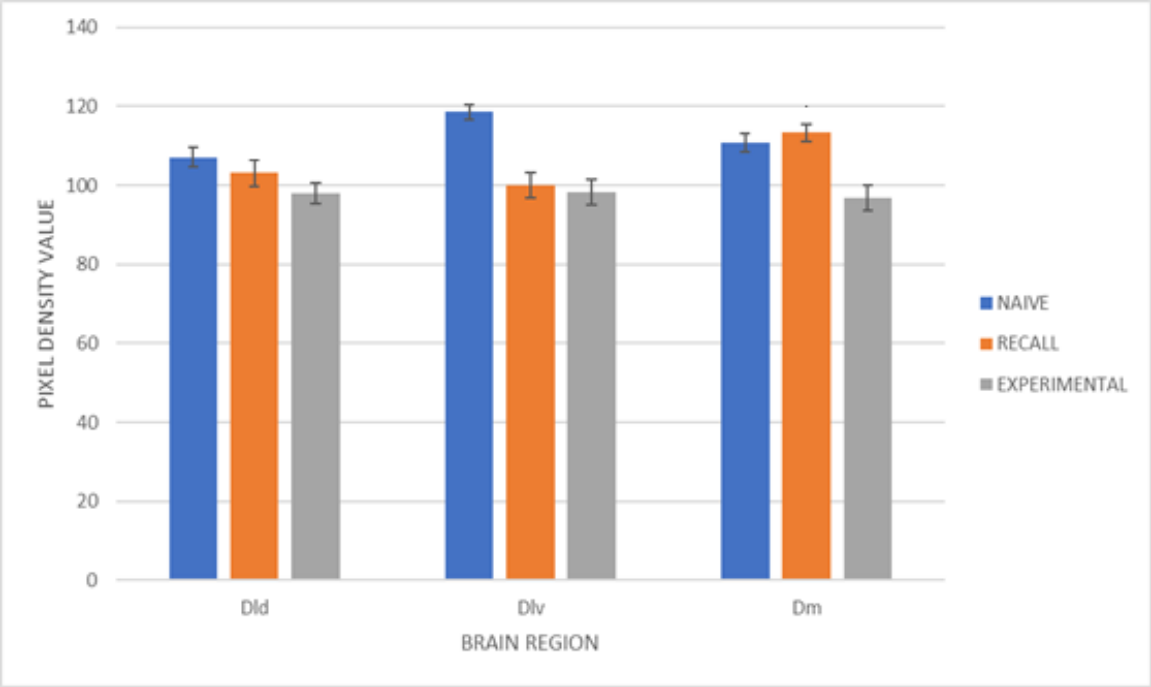

Figure 10. Pixel density values of Dld, Dlv, and Dm regions of the forebrain in the Naïve, Recall, and Experimental treatments. Lower numbers indicate higher cytochrome oxidase activity.

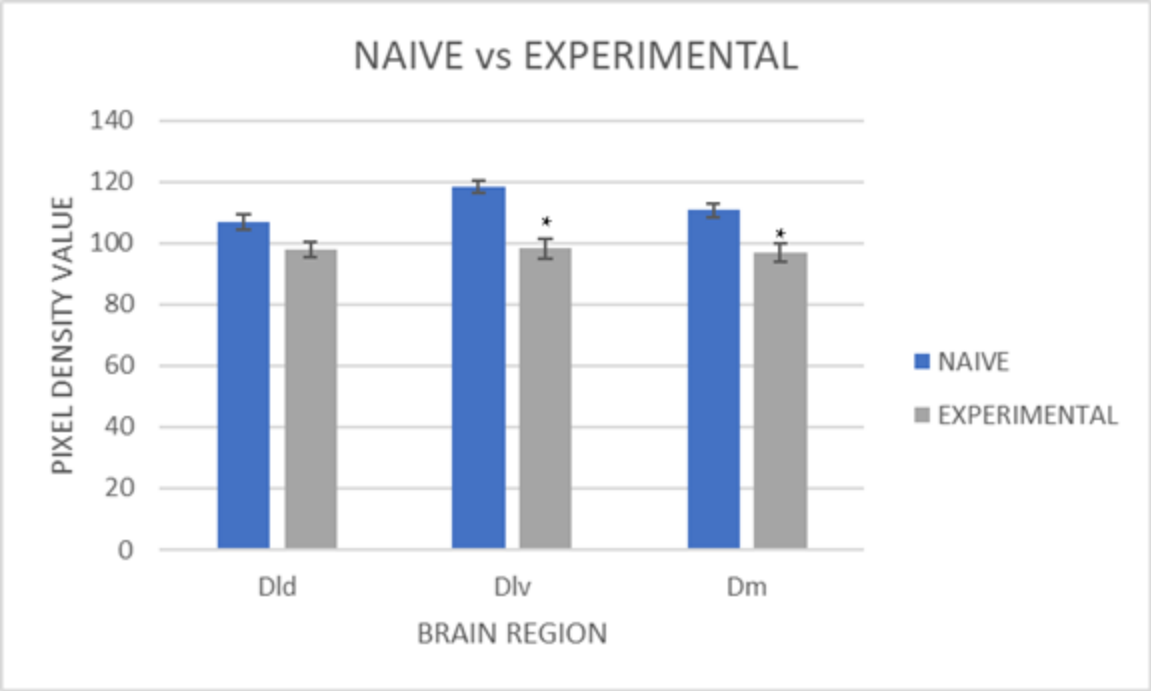

Figure 11. Comparison results of Naïve vs Experimental cohorts. Lower values indicate higher cytochrome oxidase activity.

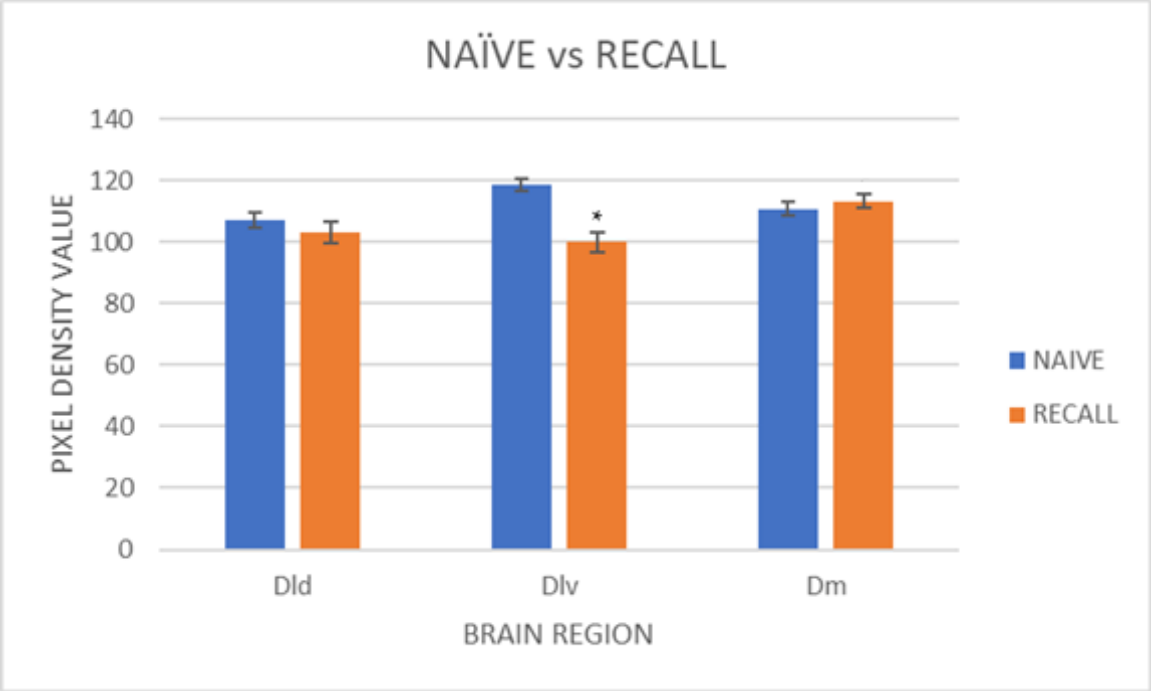

Figure 12. Comparison results of Naïve vs Recall cohorts. Lower values indicate higher cytochrome oxidase activity.

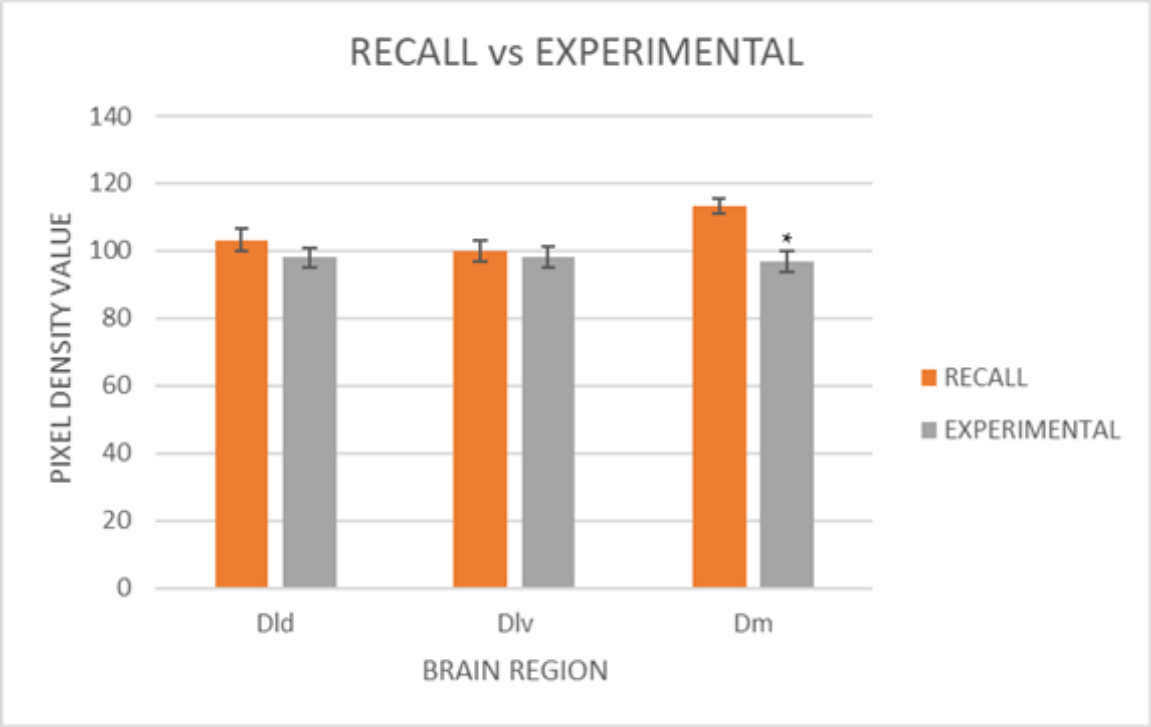

Figure 13. Comparison results of Recall vs Experimental cohorts. Lower values indicate higher cytochrome oxidase activity.

Table 1. Table with standard error for each treatment by region

REGION	N-AVG	N-STDERR	R-AVG	R STDERR	X AVG.	X STDERR
Dld	107.0375	2.47417327	103.1111111	3.343072742	97.95138889	2.696555332
Dlv	118.5666667	1.95470303	99.99305556	3.235000278	98.20138889	3.256096532
Dm	110.7916667	2.32675247	113.2575	2.296444954	96.79861111	3.140585254

Table 2. P values for comparisons of region by treatment resulting from two-way repeated measures ANOVA. Highlighted cells indicate significant differences.

TREATMENT within REGION Dld				
TREATMENTS	**MEAN DIFFERENCE**	**DF**	**P VALUE**	**SIG FLAG**
NX	-0.38194	140	0.99484	0
NR	-5.54167	140	0.34058	0
XR	-5.15972	140	0.39231	0
TREATMENT within REGION Dm				
TREATMENTS	**MEAN DIFFERENCE**	**DF**	**P VALUE**	**SIG FLAG**
NX	7.04861	140	0.017723	1
NR	-9.41028	140	0.4776	0
XR	-16.45889	140	0.000153	1
TREATMENT within REGION Dlv				
TREATMENTS	**MEAN DIFFERENCE**	**DF**	**P VALUE**	**SIG FLAG**
NX	15.20833	140	0.000506	1
NR	13.41667	140	0.00248	1
XR	-1.79167	140	0.89246	0

Table 3. Values for multivariate tests resulting from two-way repeated measures ANOVA treatment vs region. Highlighted cells indicate significance.

MULTIVARIATE TEST	VALUE	F	NUM DF	DF	P>F
PILLAI'S TEST	0.54976	9.76836	4	32	2.79E-05
WILKS' LAMBDA	0.45024	9.76836	4	32	2.79E-05
HOTELLING'S TRACE	1.22105	9.76836	4	32	2.79E-05
ROY'S LARGEST ROOT	1.22105	9.76836	4	32	2.79E-05

Table 4. Values for tests of within-subjects effects resulting from two-way repeated measures ANOVA treatment vs region. Highlighted cells indicate significance.

TEST	SUM OF SQUARES	DF	MEAN SQUARE	F	P>F
SPHERICITY ASSUMED	6457.78922	4	1614.4473	7.94448	8.43E-06
GREENHOUSE-GEISSSER	6457.78922	3.634	2108.03358	7.94448	6.92E-05
HUYNH-FELDT	6457.78922	3.39057	1904.6349	7.94448	3.31E-05
LOWER-BOUND	6457.78922	1	6457.78922	7.94448	7.88E-03

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