GENETIC MECHANISMS OF TELENCEPHALON DIVERSIFICATION THROUGH SHIFTS IN THE PALLIAL-SUBPALLIAL BOUNDARY

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GENETIC MECHANISMS OF TELENCEPHALON DIVERSIFICATION THROUGH SHIFTS IN THE PALLIAL-SUBPALLIAL BOUNDARY

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LIST OF SYMBOLS AND ABBREVIATIONS

μ-	micro (10^{-6})
m-	milli (10^{-3})
cere	Cerebellum
fb	Forebrain
hb	Hindbrain
hypo	Hypothalamus
mb	Midbrain
of	Olfactory Region
pal	Pallium
pd	Pallidum
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline, 1% Tween
PFA	Paraformaldehyde
PK	Proteinase K
pretec	Pretectum
PSB	Pallial-Subpallial Boundary
pt	Prethalamus
rhom	Rhombomere
subpa	Subpallium
teg	Tegmentum
tel	Telencephalon
thal	Thalamus
ZLI	Zona Limitans Intrathalamica

SUMMARY

The vertebrate brain develops through the formation of compartments. These compartments are physically separated to allow for the proper differentiation of each structure within the brain. The telencephalon, a compartment analogous to the cerebral cortex of mammals, further subdivides once it is separated from the rest of the developing forebrain. The first division within the telencephalon splits it into the ventral and dorsal divisions, or the subpallial and pallial regions, respectively. The pallial-subpallial boundary (PSB) separates these regions to ensure proper development of each telencephalic structure.

The pallium develops into memory storage and processing centers, and the subpallium further divides into the pallidum and the olfactory bulbs, which are involved in motor coordination and scent processing, respectively. Because of the different ecological niches occupied by cichlid species, they utilize certain telencephalic structures moreso than others and because of the space constraints, telencephalic morphology reflects these preferences. Mbuna species, which feed among the rocks scraping algae, utilize their sense of smell and have large olfactory bulbs. Non-mbuna species, which feed in the water column and utilize eyesight and possibly memory for recognition of prey, have larger pallial structures.

These differences in structures are observed early in development shortly after the telencephalon separates from the remainder of the forebrain. Upon formation of the PSB, placement and angle of the boundary are distinctly different in mbuna and non-mbuna species. In mbuna species compared to non-mbuna species, the PSB is shifted dorsally, allowing more tissue to be allocated to the developing olfactory bulbs. The PSB is shifted ventrally in non-mbuna species to allocate more tissue to the progenitor cells that develop into the memory-

processing center and structures that process visual input. These observed shifts in the developmental boundaries within the brain may provide insight into the evolution of structures such as the cerebral cortex.

CHAPTER 1

INTRODUCTION

Brain Development

Brain development begins in vertebrates shortly after gastrulation. As the anteroposterior and the dorsoventral axes are patterned, the mesoderm and ectoderm interact to form the neuroectoderm, which develops into the central nervous system (Keynes and Lumsden 1990). Fate maps have elucidated the process of patterning and migration in the neural plate, especially during the initial stages of neural patterning (Woo and Fraser 1995). More recently, later stages of neural development have been studied with specific interest in the compartmentalization of the developing vertebrate central nervous system (Kiecker and Lumsden 2005). Here we study this developmental compartmentalization in an evolutionary context using closely genetically related species to provide evidence for an alternative mechanism of the evolution of brain structures, specifically those within the telencephalon.

Compartmentalization

The vertebrate brain is divided into multiple compartments, corresponding to the various adult structures specific for certain functions (Vincent 1998). The developing brain is first divided into the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain) (Mastick, Fan et al. 1996). These regions further divide (Figure 1) to form the structures of the adult brain (Kotrschal, Van Staaden et al. 1998). The hindbrain has been extensively studied due to its very clear division into rhombomeres, which are individual segments that express their own sets of genes and contain cell lineage restricted boundaries (Wilkinson and Krumlauf 1990). Experiments using ectopic *Hox* gene expression in the

vertebrate hindbrain confirmed the importance of these rhombomeres in proper formation of structures within the head, especially specific types of neurons (Bell, Wingate et al. 1999).

Segmentation similar to that observed in the hindbrain has been observed in the forebrain, strongly corresponding to the adult structures (Mieda, Kikuchi et al. 1999). The prosencephalon divides into sections similar to rhombomeres, which Puelles and Rubenstein have thoroughly mapped and described as prosomeres (1994; 2003). These prosomeres denote the developing structures of the forebrain as they correspond to their adult fate (Figure 1): the telencephalon, the thalamus, the prethalamus, and the hypothalamus (Wullimann, Puelles et al. 1999).

Structures within the regions of the developing brain correspond to the functions in the adult (Irvine and Rauskolb 2001). Sight is processed in the mesencephalon (which develops into the optic tectum, pretectum, and other sight-processing structures) as well as some tissue from the prosencephalon (Nakamura, Sato et al. 2008). The telencephalon, analogous to the human cerebral cortex, processes information from the olfactory bulbs (at the rostral tip of the telencephalon) as well as taste information. The telencephalon is involved in behavioral functions primarily concerning learning and reproduction (Wilson and Rubenstein 2000). The thalamus (which merges later in development with the prethalamus) is largely responsible for the relaying of sensory information (Kitagawa, Watanabe et al. 2004). These different structures are separated by cell lineage restriction boundaries, and the differences in the placement of these developmental boundaries, specifically in the telencephlon, are the subject of this study.

Developmental Boundaries

Compartments within the developing brain are separated by developmental boundaries, beginning with the separation of the prosencephalon, mesencephalon, and rhombencephalon (fb, mb, hb, respectively in Figure 1). These barriers confer the fates of flanking regions by

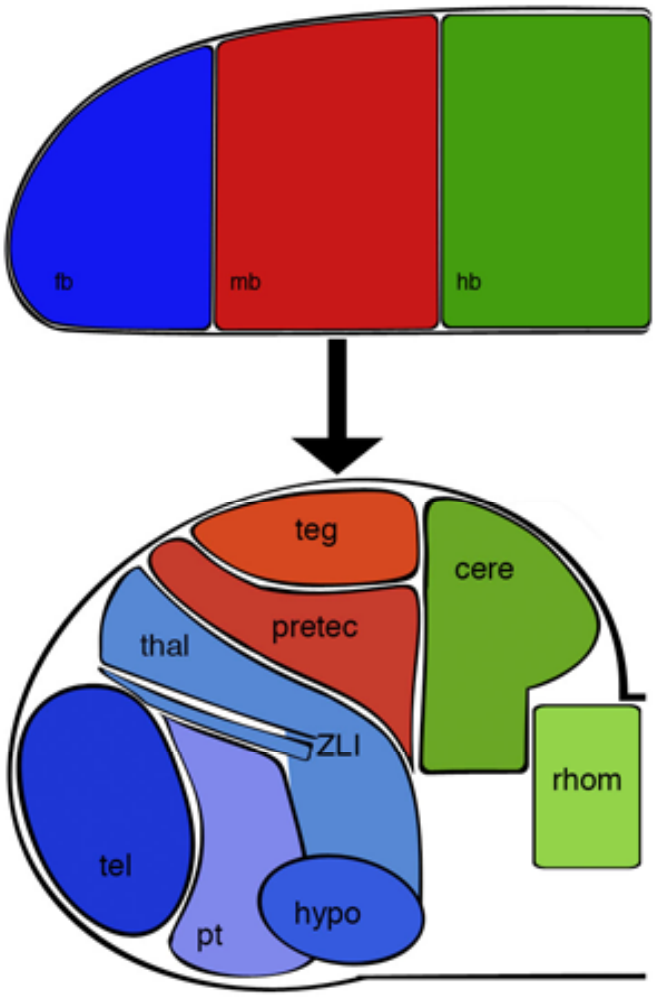

Figure 1: Segmentation of the developing vertebrate brain. The neural tube first divides into the forebrain (fb), midbrain (mb), and hindbrain (hb). Each segment further divides into sub-regions, most of which are divided by cell-lineage restriction boundaries (i.e. the zona limitans intrathalamica, ZLI). Segmentation ensures the proper differentiation of structures of the adult brain. Some sub-regions further divide (i.e. the telencephalon), while some merge to form a single adult structure (the thalamus and prethalamus merge to form the adult thalamus). tel: telencephalon, pt: prethalamus, hypo: hypothalamus, thal: thalamus, teg: tegmentum, pretec: pretectum, cere: cerebellum, rhom: rhombomere

preventing cells from crossing into other regions of the developing brain, and many primary developmental boundaries function as local signaling centers (Figure 2). Thus further specification can continue to form the separate structures found in the adult brain (Irvine and Rauskolb 2001).

Zona Limitans Intrathalamica

Within the forebrain, the zona limitans intrathalamica (ZLI) is a primary developmental boundary that plays a major role in proper development of forebrain structures as well as structures in the mesencephalon (Braun and Roelink 2001). The ZLI separates the prethalamus (ventral) from the thalamus (dorsal) through cell lineage restriction (Guinazu, Chambers et al. 2007). The compartment also serves as a signaling center by conferring differential fates onto flanking cells through *sonic hedgehog* (*shh*) expression (Vieira and Martinez 2006). Expression of *shh* confers not only the identity of region (Vieira and Martinez 2004), but also the size, based on the placement of the ZLI (Figure 2).

Pallial-Subpallial Boundary

The telencephalon, like other parts of the brain, is further subdivided once it is separated from the other regions of the developing forebrain. The primary division within the telencephalon is the pallial-subpallial boundary (PSB), a cell-lineage restricted boundary that divides the telencephalon into the ventral (subpallial) and dorsal (pallial) telencephalon (Puelles and Rubenstein 1993; Wullimann and Rink 2002). Shown in Figure 3, the pallium, marked by expression of *emx2* and *pax6*, develops into the hippocampus as well as structures involved in processing information from the optic tectum (Demski 2003; Folgueira, Anadon et al. 2004). The subpallium is marked by expression of *dlx2* and *nkx2.1* and develops into the basal ganglia,

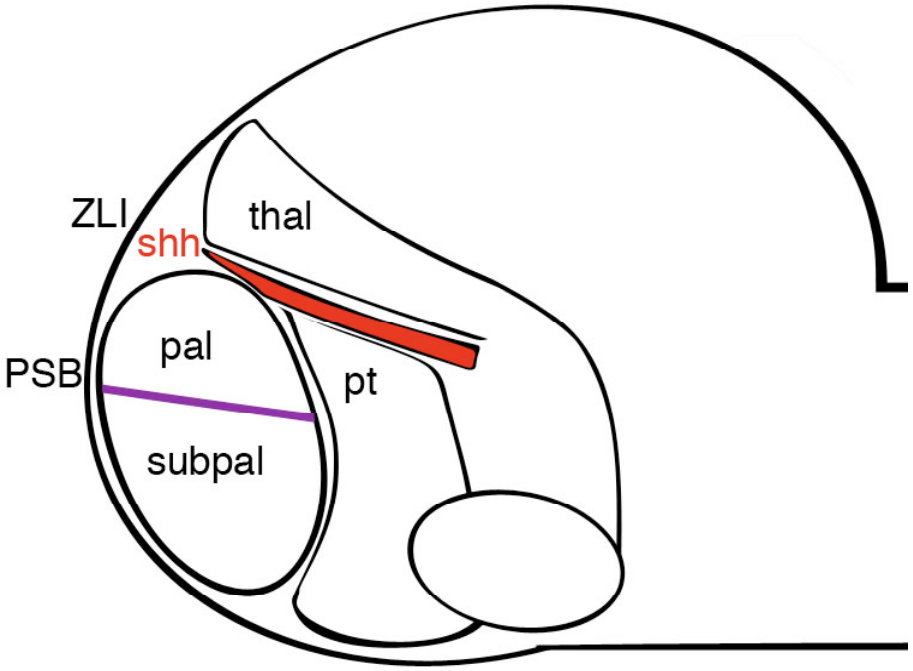

Figure 2: Developmental Boundaries in the Forebrain. The primary developmental boundary within the forebrain is the zona limitans intrathalamica (ZLI). It separates the thalamus (dorsal) from the prethalamus (ventral). Once the telencephalon separates from the rest of the developing forebrain, it begins to further divide into the structures of the adult telencephalon, starting with the development of the boundary between the pallium and subpallium, the pallial-subpallial boundary (PSB). Some of the sub-regions further divide (i.e. the subpallium), while some merge to form a single adult structure (i.e. the thalamus and prethalamus merge to form the adult thalamus). thal: thalamus, pt: prethalamus, pal: pallium, subpal: subpallium

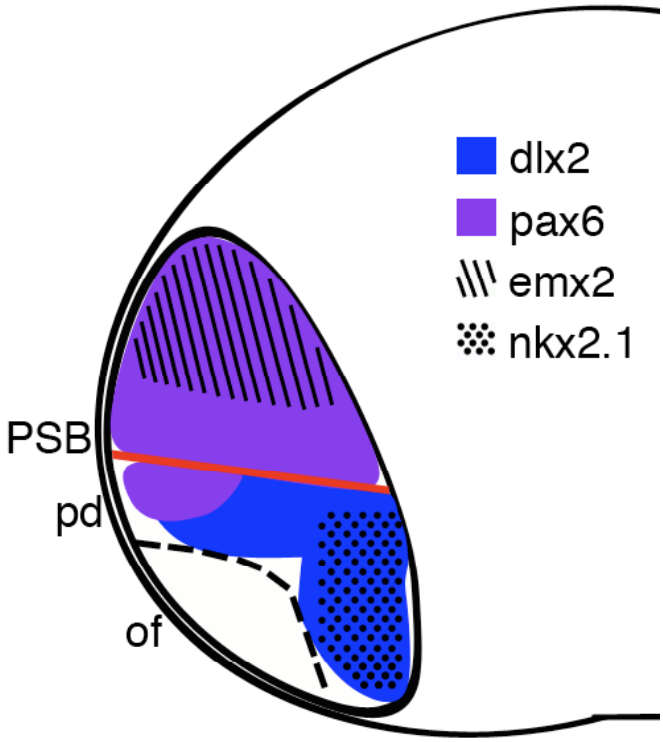

Figure 3: Telencephalic Markers. Following the emergence of the pallial-subpallial boundary (PSB, red), markers in both pallial (dorsal) and subpallial (ventral) regions are observed. *Emx2* and *pax6* are expressed primarily in the pallium, although later in development, *pax6* is developed in the subpallium approximately at the border between the pallidum (dorsal subpallium, pd) and olfactory region (ventral subpallium, of). *Nkx2.1* and *dlx2* are expressed in the subpallium. However, *dlx2* and *pax6* are the only markers that are expressed directly adjacent to the PSB.

which is responsible for coordination and planning of movement and quickly further subdivides into the dorsal pallidum and the ventral olfactory bulbs (Puelles, Kuwana et al. 2000; Folgueira, Anadon et al. 2004). Although these genes are typically studied in analyzing the PSB as well as the growth of the pallium and subpallium, in fish models only *dlx2* and *pax6* are expressed directly adjacent to the PSB. Thus the differential expression of these genes is the focus of this paper in order to make inferences about differences in the PSB.

Alterations in structures within the telencephalon have been observed in mutants for these essential pallial and subpallial markers. *Nkx2.1* mutants have a much larger pallium and thus a smaller subpallium (Sussel, Marin et al. 1999). In another study, *dlx1/dlx2* was shown to be down regulated in response to the up regulation of *pax6*, indicating a ventral shift in the PSB (Faedo, Quinn et al. 2004). However, scientists have only been able to study the significance of these genes in telencephalic development, not their possible role in the evolution of telencephalic structures. Cichlids, a group of very closely related species, provide an excellent model for the study of evolutionary processes in a natural environment.

Lake Malawi Cichlids

East African cichlids have undergone an extraordinary evolutionary radiation resulting in up to 1000 very closely genetically related species in around 500,000 years (Sturmbauer and Meyer 1992; Sultmann, Mayer et al. 1995). Vast environmental diversity in the tropical African lakes in which these species have evolved allowed for a vast number of ecological niches of which ancient cichlids could take advantage in order to lower competitive pressure (Lowemcconnell 1993). Because of the close genetic relationship of these species coupled with the extensive morphological, behavioral, and ecological diversity, cichlids from the major East African lakes (Lake Victoria, Lake Tanganyika, and Lake Malawi) serve as an excellent

evolutionary model (Meyer 1993). Modern cichlids occupy a wide range of ecological niches (at different depths and along different aquatic terrain) and consume a wide variety of food sources (Huber, vanStaaden et al. 1997).

Mbuna vs. Non-Mbuna Species

As shown in Figure 4, general ecological niches are reflected in cichlid brain morphology (Shumway 2008). Mbuna (rock-dwelling) species feed primarily along the shores, scraping algae from rocks. Because they graze along the rocky terrain of the lakes, mbuna species (largely detritivores, herbivores, and molluscivores) utilize their senses of smell and taste but do not rely heavily on sight (Genner and Turner 2005). Conversely, non-mbuna species (insectivores, paedophages, fin-biters and scale eaters, and especially piscivores) occupy the water column and actively pursue food sources. These species therefore utilize their sense of sight, and they do not depend as greatly on their senses of smell and taste (Huber, vanStaaden et al. 1997).

Zona Limitans Intrathalamica Shift

Through in situ hybridization, we have previously observed shifts in the ZLI when comparing the development of the ZLI in mbuna and non-mbuna cichlid species (Figure 5). A rostral shift in the ZLI was observed in non-mbuna species, allowing greater allocation of tissue to the thalamus, in which much of the tissue is later co-opted for midbrain (sight-processing) function. Conversely, we have observed a caudal shift in the ZLI of mbuna species, resulting in a larger ratio of telencephalon to thalamus compared to non-mbuna species.

Brain Evolution

Ecological Influences on Morphology

Environment appears to have acted upon brain morphology to help produce the wide range of species observed in the African great lakes, including Lake Malawi, by providing a

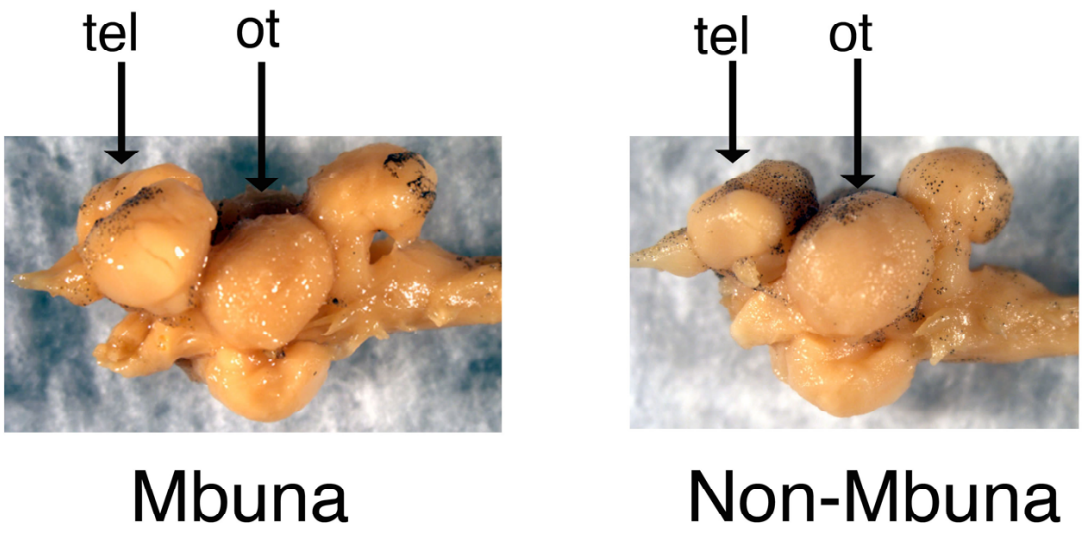

Figure 4: Mbuna vs. Non-Mbuna Adult Brains Reflect Ecological Preferences. The ecological niches of mbuna and non-mbuna species correspond to the morphology of the brains of both types of species. Mbuna species, which feed in rocky tidal areas through filter feeding, optimize the telencephalon. This area of the forebrain is involved in processing of taste and smell. These species have very large telencephala and small optic tecta. Conversely, non-mbuna species feed in the water column and visually locate their prey. These species optimize the optic tectum, responsible for sight processing, and exhibit large optic tecta with small telencephala. This may be due to a tradeoff early in development.

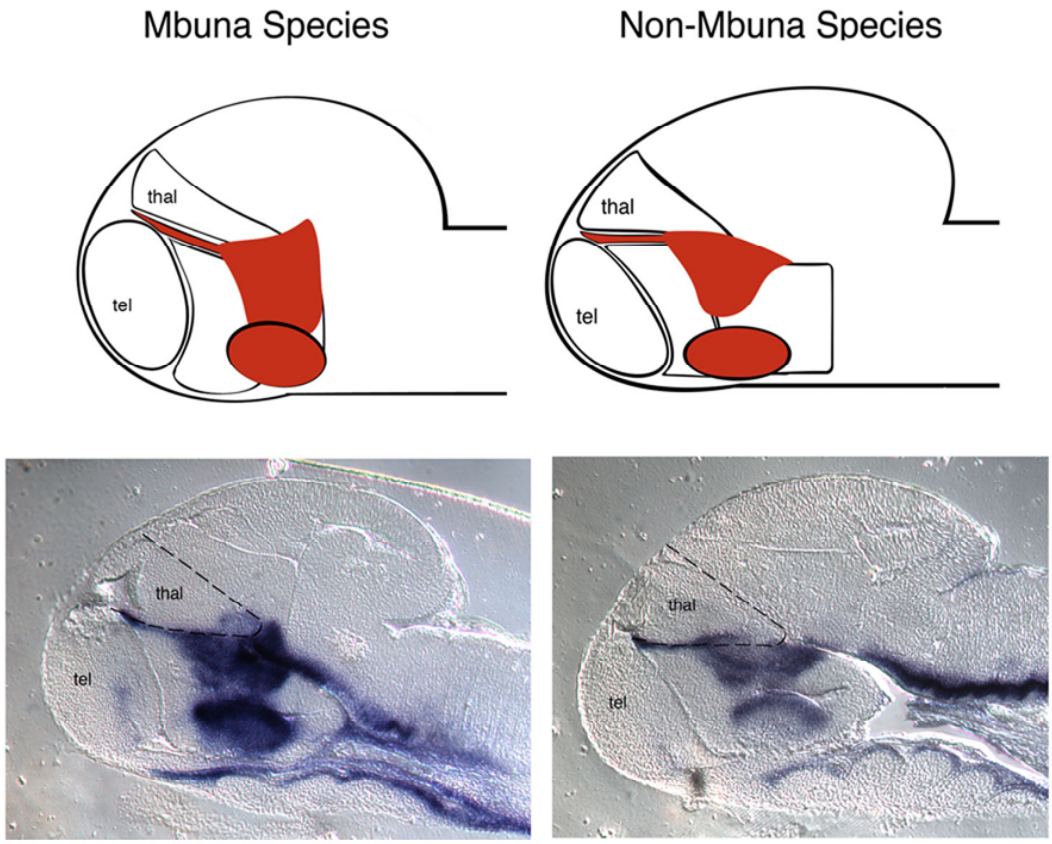

Figure 5: Shifts in the ZLI, Comparing Mbuna and Non-Mbuna Species. The zona limitans intrathalamica (ZLI, red) separates the thalamus and prethalamus, secreting *sonic hedgehog* (*shh*). The ZLI impacts flanking regions through both *shh* signaling and its position. In mbuna species, which feed among the rocks and optimize functions processed by the telencephalon, the ZLI is shifted caudally compared to that of non-mbuna species, which feed in the water column and optimize functions processed by the optic tectum/thalamic region. Measures of the developing telencephalon and thalamus reflect the impact of this shift: mbuna species have larger telencephala, while non-mbuna have larger thalami/ optic tecta.

basis for selection of brain morphologies for fish in various niches (Schliewen, Tautz et al. 1994). A wide variety of food sources and habitats allowed extreme advantage for speciesthat were best able to morphologically adapted to the specific aspects of the niche. Such physical aspects as brain morphology adapt to the resources available as ancestral species further specified their ecological niche (Fryer 1996).

The brain structures responsible for processing senses of sight and smell/taste have been seen to correlate with their relative use in mbuna and non-mbuna species (Figure 4): mbuna species have larger taste and smell processing centers (telencephala), while non-mbuna species have larger sight-processing centers (optic tecta) (Ito, Ishikawa et al. 2007). These differences have presumably arisen because the variation in resources and environment provided a basis for selection acting upon brain morphologies, which optimize different areas to enhance those senses most needed. Regions of the brain could be altered through genetic mechanisms similar to those seen in other systems, including zebrafish, birds, and mammals, especially through genetic manipulation (Pollen and Hofmann 2008).

A possible tradeoff has been recognized when comparing optic tectum and telencephalon relative sizes in mbuna and non-mbuna species. Mbuna species that optimize the telencephalon have a much smaller optic tectum compared to non-mbuna species, which have a much larger optic tectum and smaller telencephalon. This tradeoff correlates with the environmental constraints of mbuna and non-mbuna species. The smaller of the two structures is less essential for the fish's feeding and survival (Huber, vanStaaden et al. 1997).

Neurogenesis

The currently held model of brain evolution centers on the idea of neurogenesis or neuronogenesis (Caviness, Takahashi et al. 1995). This theory holds that the expansion of the

cortex in vertebrates and its changes over time are explained by changes in the regulation of genes that control the growth and death of cells, specifically those that control entrance into and exit out of the cell cycle and the type of mitosis (asymmetric or symmetric) (Rakic 1995). Studies have shown that altering the expression of these genes (i.e. β-catenin) can alter the area and size of the cortex (Chenn and Walsh 2002).

These studies have focused largely on the mammalian cortex and have concentrated on events long after the patterning of the forebrain is complete (Caviness, Takahashi et al. 1995). Because cichlids are closely genetically related, they serve as an excellent model for studying comparatively how vertebrate brains are patterned early in development (Pollen and Hofmann 2008). Studies utilizing this model could give insight into the origin of the cortex as seen in higher vertebrates such as mammals.

This study analyzes the differences in the pallial-subpallial boundary (PSB) in the telencephalon of mbuna and non-mbuna cichlid species, making connections to the differences in adult structures. Shifts in the PSB result in size differences of pallial and subpallial structures. The large cortex seen in higher vertebrates, as well as the differences in other telencephalic structures, may originate through shifts in these primary developmental boundaries and increase through neurogenesis.

Based on the known ecological niches of certain mbuna and non-mbuna species (*Metriaclima zebra*, *Labeotropheus fuelleborni*, *Copadichromis borleyi*, *Mchenga conophorus*), I expected a ventral shift in the PSB of non-mbuna species compared to that in mbuna species, resulting in a larger pallium in non-mbuna species and a larger subpallium in mbuna species. A larger subpallium in mbuna species would allow for larger olfactory bulbs to develop, while a larger pallium in non-mbuna species would allow for a great volume of tissue to be connected to

the optic tectum, enhancing overall sight processing. The early appearance of these variations during development provide evidence for the importance of patterning genes (compared to those involved in neurogenesis) in the evolution of telencephalic structures.

CHAPTER 2

MATERIALS AND METHODS

Fish Maintenance

All cultured Lake Malawi cichlid species were kept in aquariums continuously filtered and maintained at 27°C with a 24-hour cycle of light and dark to simulate natural conditions and ensure optimal activity of the species (Georgia Institute of Technology). To obtain embryos from the species considered in this paper (*Metriaclima zebra*, *Labeotropheus fuelleborni*, *Copadichromis borleyi*, *Mchenga conophorus*) lab members took note of the age of each brood (in days post-fertilization, or dpf) and at the appropriate age, squeezed the mouth of the female to dislodge and remove the embryos.

The embryos, typically ranging from 1 to 5 days and still within the chorion, were immediately fixed in 4% paraformaldehyde (PFA) diluted in phosphate buffered saline (PBS). Embryos were fixed before removal of the chorion in order to prevent damage to the fragile embryo and yolk sac. After at least 24 hours of fixation, the embryos were gradually dehydrated in methanol for storage. Dehydrated embryos were stored at 4°C until processing through in situ hybridization.

In Situ Hybridization

Probe Design

Primers were designed by first matching NCBI results for a gene of interest with sequence data from 5 cichlid species (Loh, Katz et al. 2008). The results from the cichlid search were then run through NCBI once more using the Basic Local Alignment Search Tool (BLAST) to ensure an accurate match with the exact gene of interest, and primers were constructed using Primer3 (Altschul, Gish et al. 1990; Rozen and Skaletsky 2000). Optimal primers ranged from 600 to 800 base pairs, to ensure high specificity while maintaining high binding frequency.

Probe Preparation

Polymerase Chain Reaction

Once acquired, the gene of interest was amplified using polymerase chain reaction (PCR), consisting of a mixture of GoTAQ, dH_2O, non-specific cDNA, and each of the left and right (complimentary) primers. Positive and negative control reactions were also performed. A repeated cycle of temperature changes, with the exact temperatures depending on the sequence of the primers, were run in turn for approximately 20 cycles. Gel electrophoresis using a 1% agarose gel stained with 0.5μg/mL ethidium bromide (Sigma) and 10% TBE buffer verified proper amplification of the gene of interest. Gels were viewed under ultraviolet (UV) light.

Ligation and Transformation

In order to further amplify the gene of interest and create a functional product, the PCR product was ligated into a vector and transformed into bacterial cells. Ligation was carried out by adding purified DNA to pGEM®-T Easy Vector (Promega), T4 DNA Ligase, and 2X Rapid Ligation Buffer. The ligation solution was incubated for 1 hour at room temperature, and transformed in a 1:10 dilution to *Escherichia coli* cells. The cells with ligation solution were incubated on ice for 30 minutes, heat shocked at 42°C for 60 seconds, and iced for 2 minutes. Transformed cells were incubated in LB media for 1 hour to initiate growth.

After the 1-hour incubation, cells were plated onto a dry, gently heated LB agar plate with ampicillin for selection of transformed colonies. *E. coli* colonies were allowed to grow at 37°C for approximately 24 hours, and 8 to 10 colonies were selected and plated on a replica plate. These same colonies were also run through PCR and subsequent gel electrophoresis to verify transformation of the gene of interest.

Sequencing

In order to verify the exact sequence of the plasmid transformed, plasmid DNA was sequenced. Resulting sequences were visually analyzed using Sequencher® for confidence. Sequences obtained were compared to those in the NCBI database using the BLAST tool (Altschul, Gish et al. 1990).

Plasmid Purification

A colony from the replica plate was chosen and grown in 150mL LB media for 24 hours. A QIAGEN HiSpeed® Plasmid Maxi Kit was used with the 150mL bacterial culture to purify the plasmid. This kit allows purification of a high copy of the plasmid, resulting in approximately 750μg of pure plasmid.

Digesting the Plasmid

The gene of interest was separated from the rest of the plasmid through use of restriction enzymes. If the gene was completely sequenced, then cut sites within the sequence were easily identified for each restriction enzyme. In this case, the plasmid was cut by adding a small sample of the Maxiprep to a mixture of buffer, the chosen enzymes (those which cut at each end of the gene of interest), and dH_2O. If the sequence had not been obtained, then test digestions were performed in which multiple different reactions were run with a smaller amount of Maxiprep and varying restriction enzymes. After the adequate reaction was identified through gel electrophoresis, the plasmid was cut as described above. The mixture was incubated for 2 hours at 37°C and run on an agarose gel to confirm success of the reaction based on the size of the sample.

Cleaning the Digest

After the cut reaction had completed incubation, linear plasmid was added to 3M sodium acetate (NaOAc) and 1:1 phenol/chloroform to begin cleaning the digest. After centrifugation, the aqueous phase was taken and 2.5 times the volume EtOH was added. The mixture was once again centrifuged and the aqueous phase was discarded. The pellet was rinsed for 2 minutes in 70% EtOH then redissolved in dH_2O.

Adding cleaned linear DNA to 5X buffer, DTT, DIG mix (Roche), RNase inhibitor (Roche), RNA polymerase, and dH_2O initiated the T7 and Sp6 reactions, the sense and antisense directions. The mixture was incubated in a water bath at 37°C for 2 hours, and DNase was added to leave only the RNA product. The mixture was once again incubated for 15 minutes, and dH_2O, LiCl, and 100% EtOH were added to initiate probe precipitation. The mixture was kept at -20°C for approximately 1 hour and centrifuged. The aqueous phase was discarded and the pellet was washed with 70% EtOH. The pellet was allowed to dry and finally resuspended in dH_2O. The completed mixture was kept at -20°C until use in the in situ hybridization protocol.

In Situ Hybridization Protocol

In situ hybridization was carried out according to the protocol published by Dr. Gareth Fraser (Fraser, Graham et al. 2004), adapted from the protocol by Xu et al (Xu, Holder et al. 1994). The embryos that were dehydrated in methanol and kept at -20°C for at least 24 hours were rehydrated gradually in PBST (PBS with 1% tween): embryos were washed for 10 minutes in 25%, 50%, then 75% methanol in PBST, and finally in 100% PBST. After rehydration, the embryos were de-chorionated, during which the chorion was removed either only from the head-region of the embryo or from the entire embryo and yolk sac. The former was preferred in order to maintain the connection of the embryo to the yolk sac.

After the embryos were de-chorionated, the embryos were digested in proteinase K (PK) to lyse cells in the embryo, allowing the probe to later reach cells within the embryo. PK was diluted in PBST in a 1:1000 dilution using 10μg/mL stock PK. The dilution was multiplied by the age in days of the embryo, such that a 4-day-old embryo was digested in a 4:1000 dilution of PK. Embryos were taken through the rest of the *in situ* hybridization according to the specifications of the protocol, where the reactions took place in a 24-well plate. The embryos were divided among the wells according the species/age and gene of interest. The ending color reaction generated by the activation of alkaline phosphatase was monitored visually until satisfactory staining had been achieved, at which point the color reaction was stopped by rinsing in PBST and fixing in 4% PFA. The embryos were stored in PBS at 4°C.

Whole Mount Imaging

Stained embryos were first photographed in whole-mount. Embryos were placed on agarose medium in a Petri dish with PBS to be easily oriented and visualized using a Leica MZ16 stereomicroscope. Images were photographed using a Leica digital camera attachment and edited using Adobe Photoshop v 9.0.

Sectioning and Imaging

Afterward whole-mount visualization and processing, the embryos were set in 800μL gelatin-albumin, made by dissolving 2.2g gelatin in 450mL PBS; 135g chick egg albumin was added under a fume hood and after it had dissolved, 90g sucrose was added. The stock was stored -20°C in 15mL aliquots. The embryo was fixed in the gelatin-albumin solution by adding 80μL 2.5% gluteraldehyde and adjusting the embryo according the orientation of the section desired. The blocked embryos were stored in quart-sized polyethylene plastic bags with a moist paper towel at 4°C overnight to fully set. The following day the embryos were fixed in 4% PFA then stored in PBS. The finished blocks were oriented sagittally or frontally and mounted on the

stage of a Leica Vibratome VT1000 using cyanoacrylate glue. Sections with a thickness of 15μm were collected and placed onto glass slides. One drop of 90% glycerol/PBS was added and a glass cover slip was placed onto the slide. Specimens were visualized under a Leica DM2500 microscope at 10X magnification and photographed using an attachable Leica digital camera. Images were analyzed using Adobe Photoshop v 9.0.

CHAPTER 3

RESULTS

Whole Mount Imaging

After in situ hybridization was complete, embryos were photographed to ensure proper orientation of sectioned embryos. Individual differences were noted, but could not be further specified or quantified until after the embryo was sectioned. Whole mount pictures also allowed for a more complete view of the embryo, especially in cases of multi-dimensional gene expression differences among species.

Dlx2

As seen from both whole mount and sectioned embryos, *dlx2* is visibly expressed in the forebrain embryos as early as 1 day of age, or approximately 10 somites. Gene expression is observed in the telencephalon upon formation of the boundary between the telencephalon and the developing forebrain. From a sagittal viewpoint at the midline, telencephalic expression at 2 days forms a narrow strip along the ventral side of the PSB, and during and after 3 days expression forms the shape of a butterfly wing, with the dorsal finger bordering the ventral side of the PSB. From a frontal view, telencephalic expression reflects that seen in sagittal sections but greater elucidates the inverse- V-shape of the PSB observed in the lateral to medial sagittal sections.

In mbuna species compared to non-mbuna species, *dlx2* expression in the telencephalon is significantly different in shape and position. In mbuna species the dorsal finger bordering the dorsal side of the developing olfactory bulb is much thicker than that in non-mbuna species. The angle of the dorsal finger of gene expression relative to the dorsal telencephalic boundary is greatly decreased in mbuna species compared to non-mbuna species. From a frontal viewpoint,

dlx2 expression extends much farther dorsally in mbuna species compared to non-mbuna species (Figure 6).

Pax6

Expression of *pax6* is visible in the forebrain as early as gastrulation. After the telencephalon separates from the rest of the forebrain strong expression is observed in 2 spots within the subpallium, in the lateral portions of the pallidum bordering the developing olfactory bulb. Much lighter expression is observed in the entire pallium at around 3 days. Expression further fades in the pallium as the embryo develops.

In mbuna species *pax6* expression is greatly decreased relative to the remainder of the telencephalon compared to non-mbuna species. From a frontal view, expression of *pax6* differs between mbuna and non-mbuna species in that expression extends more ventrally in non-mbuna species compared to mbuna species. Expression in the subpallium also differed between mbuna and non-mbuna species. While *pax6* expression in the pallium extends more ventrally in non-mbuna species, the expression in the pallidum within the subpallium is observed more dorsally (Figure 6).

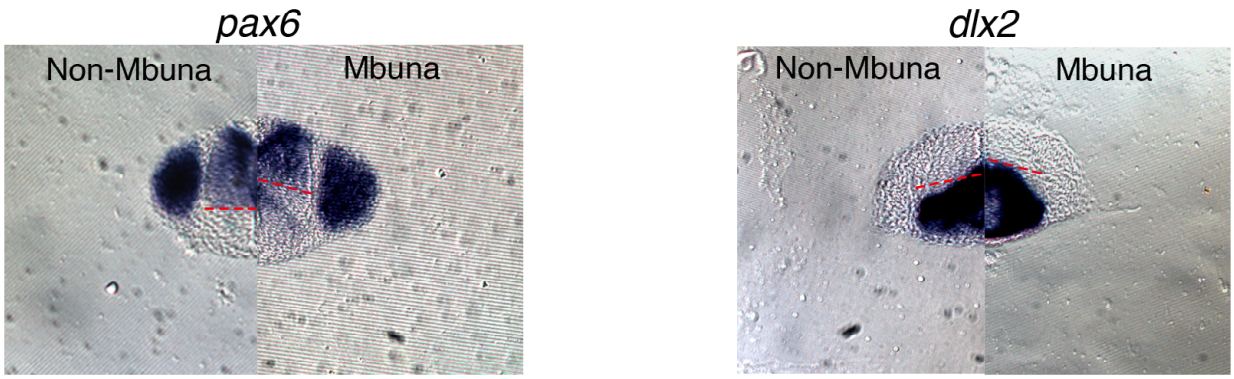

Figure 6: PSB shifts based on pallial and subpallial marker expression. Pallial marker *pax6* expression extends farther ventrally in non-mbuna species compared to mbuna species, corresponding to farther dorsal expression of subpallial marker *dlx2* in mbuna species. Because *pax6* and *dlx2* are expressed directly adjacent to the PSB (red dotted line), this differential expression indicates a shift of the PSB ventrally in non-mbuna species and dorsally in mbuna species. Sections were matched based on distance from the posterior end of the telencephalon, as sections analyzed were immediately before the end of the telencephalon marked by the optic chiasm. Differences in exact patterns of expression and location within the eye are explained by species differences. Mbuna species typically have larger olfactory bulbs, reflected in *pax6* expression in the subpallium of the mbuna species abutting the developing olfactory bulbs. Mbuna species also generally have eyes positions farther posteriorly than non-mbuna species, which is observed in the above sections.

CHAPTER 4

DISCUSSION

Shift in the PSB

The observed differential gene expression implicates a shift in the pallial-subpallial boundary (PSB). In mbuna species compared to non-mbuna species, expression of pallial markers is greatly decreased, and expression of subpallial markers extends further dorsally, indicating a dorsal shift in the PSB. This allows more progenitor cell tissue to be allocated to the subpallium, resulting in a larger subpallial region in the adult cichlid, especially the olfactory bulbs. Conversely, non-mbuna species show a ventral shift of the PSB (Figure 7). The adult non-mbuna species accordingly show a larger pallial region of the telencephalon.

The angle of the PSB is shifted in mbuna species compared to non-mbuna species. This shift is especially evident in younger embryos (approximately 2 days), when a cell lineage restriction boundary forms between the telencephalon and the rest of the developing forebrain. As the embryo continues to develop, the angle of the PSB relative to this boundary in both mbuna and non-mbuna species increases, but maintains the difference seen in younger embryos. Before the differential expression of neurogenesis genes, the angle of PSB in non-mbuna species is observed to be much less (approximately 45°) than that observed in mbuna species (approximately 90°). Neurogenesis results in the eversion of the telencephalon in ray-finned fishes (Northcutt, Neary et al. 1978), such that medial telencephalic tissue becomes lateral but dorsal and ventral differences are maintained in the positions observed in younger embryos.

Substantial evidence for significant differences in the size of the pallium and subpallium was gathered comparing mbuna and non-mbuna species. The pallium, marked by *pax6* expression, is larger in non-mbuna species, indicated in frontal sections. Conversely, mbuna

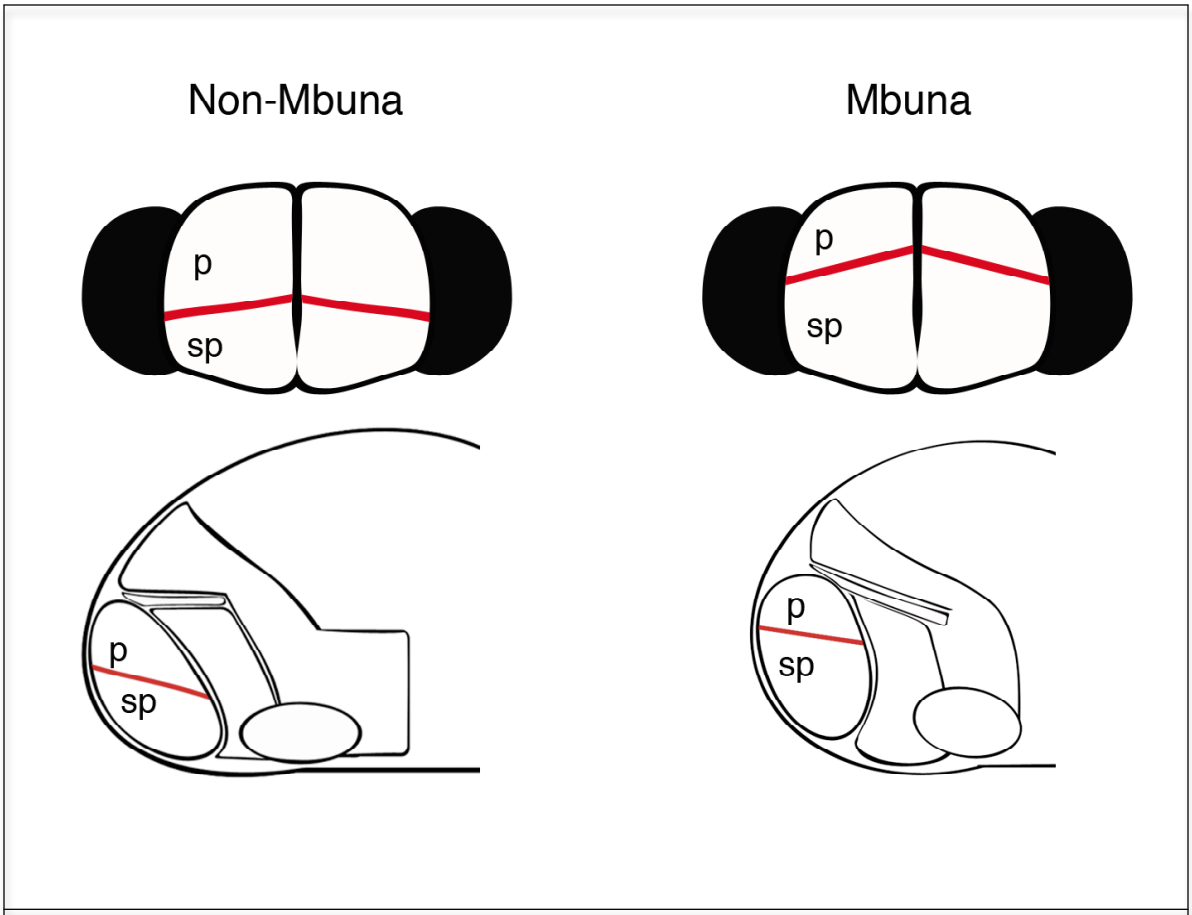

Figure 7: Shift of the Pallial-Subpallial Boundary in Mbuna vs. Non-Mbuna species. A shift in the PSB (red) is inferred based on the differential expression of *dlx2* and *pax6* while comparing mbuna and non-mbuna species: *dlx2* expression extends more dorsally in mbuna species, while *pax6* is expressed in a smaller area of the pallium, resulting in a more ventral boundary of expression compared to non-mbuna species. Because these genes are expressed at the boundary of the PSB, this differential expression indicates a shift in the PSB. p: pallium, sp: subpallium

species exhibit a larger subpallial region, marked by *dlx2* expression. Expression patterns of *emx2* and *nkx2.1* were not evaluated in this case because a) expression patterns of both genes arise after *pax6* and *dlx2* and b) neither gene is expressed directly adjacent to the PSB. *Dlx2* and *pax6* are possibly more critical in patterning of the telencephalon compared to *emx2* and *nkx2.1*.

Ecological Implications of a Shift in the PSB

The structures that develop from the pallium serve primarily in processing information from the optic tectum as well as memory storage, and the subpallium develops into the pallidum (which develops into structures responsible for motor coordination) and the olfactory region (involved in smell processing). Considering the ecological niches of mbuna and non-mbuna species, it would be assumed that non-mbuna species have larger pallial regions because of the advantages associated with greater connection between the thought-processing telencephalon and the sight-processing optic tectum. Conversely, it would be assumed that mbuna species would have larger subpallial regions in order to allocate more tissue to the olfactory region, because of their heavy reliance on taste and smell in filter feeding.

The resulting proportion of telencephalic structures reflects this intuition. Mbuna species have smaller pallial regions, while non-mbuna species have smaller subpallial regions. This is largely due to the size constraints within the telencephalon. Mbuna species greatly utilize their sense of smell, and in the adults the size of the olfactory bulbs of mbuna species is vastly greater than that of non-mbuna species. This greater necessity for the sense of smell likely outweighs a need for memory processes. For non-mbuna species, the sense of smell is less heavily relied on, especially compared to the need to have greater sight processing. Thus it is more advantageous to allocate more tissue to pallium than to the subpallium.

CHAPTER 5

CONCLUSION

The observed shift in the pallial-subpallial boundary in mbuna and non-mbuna species may be a reflection of the observed shift in the zona limitans intrathalamica (Figure 5, Figure 8). The ZLI secretes *shh*, which impacts downstream genes, including *pax6* and *dlx2* as well as a number of other patterning genes. In mbuna species, the ZLI is shifted caudally (posteriorly), and the PSB is shifted similarly dorsally. Conversely, in non-mbuna species, the ZLI is shifted rostrally (anteriorly) and the PSB is shifted ventrally/rostrally. The strong reflection of the ZLI shift in the observed PSB shift is likely due to gene interactions, overall due to the shift in the ZLI or another, earlier developmental boundary. Other implications and sources of the shift in the ZLI as well as the shift in the PSB must be investigated.

The idea of neurogenesis as an evolutionary model holds that embryos are patterned the same way in related species (i.e. cichlids), and that genes involved in regulation of cell cycle exit are largely responsible for the evolution of brain structures (Caviness, Takahashi et al. 1995). Studies that have examined the possible role of patterning genes in evolution have used knockout and knockdown studies, resulting in complete destruction of the brain (Chenn and Walsh 2002). However, the use of cichlids as an evolutionary developmental model allows scientists to study closely related species and attempt to elucidate how different structures evolve. This study shows that differential expression of patterning genes may provide a basis upon which neurogenesis acts to further enhance morphological differences.

A shift in the PSB reflects the differences in ecological niches of mbuna and non-mbuna cichlid species. The dorsal shift observed in mbuna species likely corresponds to the allocation

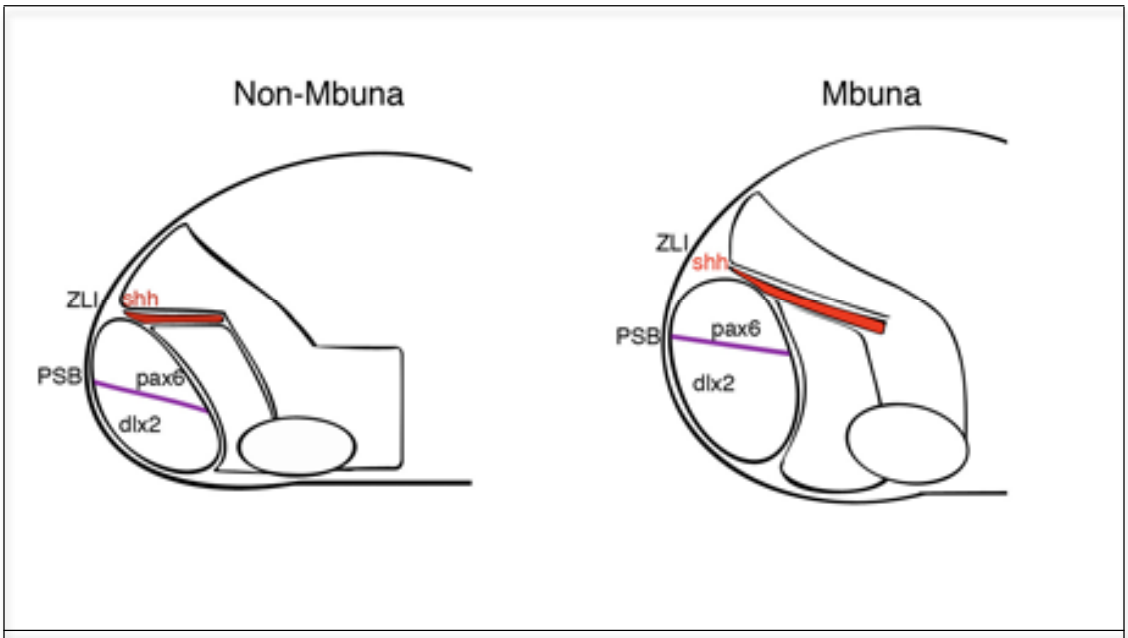

Figure 8: Impact of shifts in the ZLI on the PSB. A shift in the ZLI (red) has previously been observed when comparing mbuna and non-mbuna species. This corresponds strongly to the shift in the PSB (purple) of mbuna species compared to non-mbuna species. Because of the strong relationship between the observed shifts, the ZLI likely has some significant influence over the positioning of the PSB through some genetic pathway.

of more tissue to the developing olfactory bulbs. The pallial region in higher vertebrates develops into the cortex, which makes up the vast majority of the brain in such organisms as mammals. The shift in the PSB observed in cichlids may give insight into the origin of the notably sized cortex in other vertebrates. A gradual shift of these primary developmental boundaries, both in the brain (i.e. that between the forebrain and midbrain) and within specific areas of the brain (i.e. the PSB in the telencephalon) may give rise to the different structures upon which neurogenesis acts to produce the complex structures such as the cortex.

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