PROLIFERATION, MIGRATION, AND SURVIVAL OF CELLS IN THE

TELENCEPHALON OF THE BALL PYTHON, *PYTHON REGIUS*

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Thomas B. Bales

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TITLE: Proliferation, migration, and survival of cells in the telencephalon of the ball python*, Python regius*

AUTHOR: Thomas B. Bales

DATE SUBMITTED: June, 2014

COMMITTEE CHAIR: Christine R. Strand, PhD
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Emily Taylor, PhD
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Jason M. Blank, PhD
Assistant Professor of Biological Sciences

ABSTRACT

Proliferation, migration, and survival of cells in the telencephalon

of the ball python, *Python regius*

Thomas B. Bales

Reptiles exhibit neurogenesis throughout the brain during adulthood. However, very few studies have quantified telencephalon-wide neurogenesis in adulthood, and no studies have performed these investigations in snakes. Quantifying neurogenesis in the adult snake is essential to understanding class-wide adult neurogenesis and providing insight into the evolution of this trait. The thymidine analog 5-bromo-2'-deoxyuridine (BrdU) was used to quantify cell proliferation, migration, and survival in the ball python (*Python regius*). First, to determine the proper dose of BrdU for injection we subcutaneously injected 50mg/kg, 100mg/kg, and 250mg/kg into 15 adult male *P. regius*. We found the 250mg/kg dose marked significantly more cells than the 50mg/kg dose, but not the 100mg/kg dose. Then we subcutaneously injected 100mg/kg BrdU into 15 juvenile male P. regius at 3 different time points (2 days, 2 weeks, 2 months) prior to sacrifice to quantify proliferation, migration, and survival of cells in several telencephalic subregions. After sectioning and immunohistochemical staining, we found proliferation to be highest in the accessory olfactory bulb (AoB), retrobulbar regions (AD, AV), dorsal ventricular ridge (DVR), and dorsolateral amygdala/lateral amygdala (DLA/LA). Of the proliferating cells, the proportions of cells that migrated after 2 weeks were highest in the ventral lateral region (VL), anterior medial and lateral cortices (aMC, aLC), and anterior NS (aNS). After 2 months, the highest proportional survival was in the AoB, aLC, aMC, aNS, DVR, and ventral medial regions (VM). Regions involved in long-term functions like spatial memory may require less proliferation and longer survival, while regions involved in short-term functions undergo more proliferation with higher relative attrition.

Keywords: Python, snake, neurogenesis, BrdU, proliferation, telencephalon, CNS, brain, evolution, comparative.

ACKNOWLEDGMENTS

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Chapter 1. The use of pythons for studying adult neurogenesis

Introduction

This chapter is a comprehensive review of the literature pertaining to comparative neurology of the adult ball python, *Python regius*. First, I will provide information about *P. regius* in the laboratory and their natural habitat with a brief overview of their unique peripheral sensory systems. Then, I will discuss the evolution of the python and review past studies of the python central nervous system (CNS). The rest of this chapter will cover various aspects of adult neurogenesis, providing an overview of past adult neurogenesis research and techniques used for studies and the use of reptiles in adult neurogenesis studies, including information regarding the remarkable ability of the reptile CNS to regenerate. Lastly, I will comment on the future of research on adult neurogenesis in reptiles and include proposals for future python studies.

1. *Python regius* in the laboratory

Python regius (commonly known as "Ball Pythons" or as "Royal Pythons") are not regularly used in the laboratory for physiology studies. This is unexpected because *P. regius* are ideal organisms for laboratory study. Due to considerable use in the international exotic pet trade, they are typically byproducts of captive breeding for several generations. This process reduces the variation in the developmental environment and genetic diversity, potentially improving the internal validity of

experiments. Instead of requiring labor-intensive strategies for capturing them in the wild, they can be purchased from online retailers and can be easily maintained in cages. They are very docile and easy to handle without risk of injury to the handler or the snake. They do not require intricate laboratory housing and can be maintained by giving them access to water *ad libitum*, a heating device, and regular access to feeder mice. In fact, contrary what many researchers might expect, pythons are easier to handle and maintain than rats (Secor et al., 2001).

Yet there are also drawbacks to using snakes and other reptiles in the laboratory. Genetic and developmental laboratory studies of reptiles have been hindered by the class's slow maturation rates, ectotherm metabolism, habituation needs, and seasonal reproduction. Studies of the cellular and molecular evolution of development in reptiles are limited by the technical difficulties of embryonic manipulation (Nomura et al., 2013a). Even with these hindrances, *P. regius* has the potential to be an excellent laboratory study species. Considerable laboratory research has been done on several reptile species including anoles (Greenberg et al., 1984) and garter snakes (Morris and Crews, 1990). These studies can help provide points of comparison to prompt future examination of neurophysiology in *P. regius*.

Studies of *P. regius* are beneficial to comparative research because snakes have so many distinctive traits. For example, snakes are limbless, so comparisons of brain physiology between snakes and limbed reptiles may provide insight into the evolutionary development or loss of brain regions responsible for limb control and

motor function. Snakes are missing motor neurons at the limb level of the spinal

cord. Instead a single medial motor column developed along the entire spinal cord

(Fetcho, 1987). The rubrospinal tract is also missing in some snakes such as the

boidae (ten Donkelaar, 1988). *Python regius* also have special sensory systems

(e.g. vomeronasal, pit organ/infrared, and completely internalized hearing), for which

there have been virtually no reptilian studies of telencephalic neurogenesis (Font et

al., 2012).

Most pythons are constricting, sit-and-wait predators (Enok et al., 2012).

Since they consume large prey relative to their body size, several of their

physiological systems undergo metabolic changes, including the digestive and

cardiovascular systems (Andersen et al., 2005; Secor and Diamond, 1998). Some

species of female pythons will also undergo shivering thermogenesis. In order to

keep their eggs warm during brooding, female pythons will shiver, moving from a

series of muscular contractions of the whole body thus generating heat (Harlow and

Grigg, 1984; Stahlschmidt and DeNardo, 2008; 2009; 2010; Vinegar et al., 1970). In

order to determine the role the CNS plays in these systems, it is important to do

preliminary studies of neuroanatomy and neuroplasticity, and then perform

experiments modifying these systems to determine their effect on the brain and vice

versa.

Recently, there has been progress in understanding reptilian cognition and

intelligence, which can provide insights into the evolution of cognition, social

function, and the CNS. Behavioral laboratory studies of reptiles are very challenging,

because the classic behavioral studies are modeled on stimulus-reward, a practice not easily applied to large ectothermic reptiles that eat only every few weeks. Several researchers have attempted cognitive laboratory studies in reptiles, which have supported, at minimum, limited memory and learning (Baird Day et al., 1999b; Manrod et al., 2008; Wilkinson et al., 2007; Wilkinson et al., 2010). As examples, Gaalema (2011), using *Varanus rudicollis* (rough-necked monitor lizards), paired either black or white 5cm disk stimuli with reinforcement (thawed pinkie mice). *V. rudicololis* not only successfully learned to discriminate between the two colors, but also successfully learned two reversals, with the second reversal requiring fewer sessions than the first. Davis and Burghardt (2011) discovered that *Pseudemys nelsoni* (the Florida redbelly turtle) can observe conspecifics using visual object cues to obtain food, and then perform the task of using cues themselves to feed, even when the conspecific demonstrator is absent, regardless of spatial position. As these studies become more refined and accepted we may be able to do comparative studies of learning behavior, neurogenesis, and neuroplasticity.

2. *Python regius* in its native and altered habitat

Little is known about African pythons including *P. regius* in their native habitat (Luiselli et al., 2007; Luiselli and Angelici, 1998; Starin and Burghardt, 1992). *Python regius* has been most researched in Togo (Aubret et al., 2005a; Aubret et al., 2003; Aubret et al., 2005b; Aubret et al., 2005c), Ghana (de Buffrenil, 1995; Gorzula et al., 1997) and Nigeria (Luiselli, 2006; Luiselli et al., 2007; Luiselli and Angelici, 1998).

They are widely distributed in western Africa and abundant in southern Nigeria (Fig. 1).

Figure 1: Distribution of *P. regius* in western Africa highlighted in green.

Python regius are habitat generalists, well adapted especially to disturbed forest areas like plantations and farmland (Luiselli et al., 2007). Butler and Reid (1986) considered *P. regius* to be among those snakes with the highest degree of habitat generalism in the forested regions of southeastern Nigeria. In fact, *P. regius* are challenging to find in their unaltered habitat, and deforestation has allowed for an increase in their population size (Gorzula et al., 1997). It is also likely that *P. regius*

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are more abundant than they are predicted to be from surveys, especially those in heavily forested areas (Barker and Barker, 2006).

Above-ground activity in *P. regius* peaks and remains continuous during the wet months (June-August) and then is comparatively reduced during the dry season (Luiselli et al., 2007). Most observations of these snakes are during nocturnal hours; they are rarely seen during mid-day, when they are resting in burrows during both the wet and the dry seasons. In Togo, they are in very high density, especially in fields where rodents are present, and both males and females spend most of the daytime in rodent burrows or termite mounds, where females also protect and incubate eggs (Aubret et al., 2005a).

Female *P. regius* attain significantly larger body sizes than males, which is associated with their ability to lay 3-11 large, leathery eggs per clutch (most commonly 4-6) (Barker and Barker, 2006). Typically the female incubates her eggs underground, where they hatch after 55 to 60 days (Luiselli et al., 2007). While parental care of eggs is rare among squamate reptiles, it is ubiquitous in the python lineage, as female pythons coil tightly around their eggs throughout incubation and shiver to generate warmth (Hutchison et al., 1966).

Both age and weight are involved in determining when a python is capable of breeding. Males reach sexual maturity at 6–18 months, and for females sexual maturity is reached at 12–36 months. Males will breed at 500 grams or less, but in captivity they are often not bred until they reach approximately 800 grams; females will begin breeding at 800 grams, though 1200 grams or more is most common.

Parental care of the eggs ends once they hatch and the females leave their offspring (McCurley, 2005).

Python regius are long lived and typically grow to exceed 1m in length. Several have been captive for over 30 years in U.S. zoos (Barker and Barker, 2006), and the record for longest lived snake in captivity exceeds 47 years (Conant, 1993). When Gorzula et al. (1997) conducted a field survey of 206 adult specimens in Ghana, the range in total length was from 83.9cm to 185.9cm. The average total length of all male and female specimens at 3 years was 125cm. These populations can, although rarely, reach lengths exceeding 185cm (Cansdale, 1961).

Python regius are primarily terrestrial and climb frequently. They have also been observed spending substantial time in water, but to date, their overall ecological water presence remains unquantified. The diet of *P. regius* consists almost entirely of homeothermic prey, which is typical of other pythons of varying sizes (Luiselli et al., 2007; Shine et al., 1999; Shine et al., 1998). They consume a large proportion of arboreal species, mostly birds, in southern Nigeria (Luiselli et al., 2007; Luiselli and Angelici, 1998).

3. Sensory systems of *Python regius*

Contrary to popular belief, snakes are not deaf, even though they lack an external ear opening. Snakes, including *P. regius*, have a well-developed inner ear, even though they have lost a functional outer and middle ear (Christensen et al., 2012). In order for snakes to form a gape to consume large prey items, the mandibular tips of snake jaws must be able to move freely. In turn, they must be

stabilized by a complex muscular system. Snakes have evolved to hypertrophy these muscles, which has excluded the tympanic cavity, making the snakes' hearing ability inferior to that of other reptiles (Berman and Regal, 1967). Christensen et al. (2012) measured evoked potentials dorsal to the VIIIth cranial nerve and the brainstem in response to a wide range of frequencies and found they have lost effective pressure hearing, but have maintained or developed vibration sensitivity via sound-induced head vibrations.

Reptiles, including snakes, possess a Jacobson's organ for vomeronasal chemical sensing (mainly for detecting pheromones and prey chemoattractants). Distinctively, only sceroglossans (skinks, gekkos, varanoids, amphisbaenids, mosasaurs, and snakes) perform tongue-flicking in order to bring chemical stimuli to their Jacobson's organ from the environment. Snakes may have the most developed vomeronasal system, and are considered models for determining the structure, function, and behaviors involved with vomeronasal sensing and processing (Lanuza and Halpern, 1997b).

Furthermore, a select group of snakes, in which pythons are included (pit vipers, pythons, and boas), possess pits (containing what is commonly referred to as a "pit organ") which serve as very sensitive infrared detectors, linked to the CNS via the trigeminal ganglion (Molenaar, 1978a; Molenaar, 1978b). Information from labial pits synapses at the visual center of the brain, thus they act as infrared vision sensors allowing for the python to "see" in the infrared spectrum (Goris, 2011; Kobayashi et al., 1992).

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4. Evolution and phylogenetic analysis of *P. regius*

Python regius has been placed in the Linnaean classification: *Animalia Chordata Vertebrata Reptilia Squamata Serpentes Pythonidae Python regius*. The order *Squamata* includes lizards (~4770 species), snakes (~3000 spp.), and amphisbaenians (~170 spp.) Snakes can be organized into two main groups: Fossorial scolecophidians (blindsnakes and threadsnakes, ~340 sp.), and Alethinophidians (all other snakes, ~2640 sp.) (Heise et al., 1995; Vidal and David, 2004). *Python regius* belongs to Alethinophidia, which diverged from scolecophidians approximately 150MYA, and is typically divided into caenophidians (advanced snakes, ~2470 sp.), also known as venomous snakes, and henophidia, which includes several other groups that utilize constriction. Pythonidae belongs to the constrictive group, of which *Python* is one of the 8 genera, and *regius* is one of the 33 species (Vidal and David, 2004). The evolutionary history of snakes is controversial. Currently, it is expected they are secondly-most basal to lizards (Bruce, 2007). Pythons are the most basal extant macrostomatan species (Castoe et al., 2011; Slowinski and Lawson, 2002), thus, *P. regius* may provide insight into the evolutionary origins of adult neurogenesis and the evolution of brain morphology.

5. Evolution, anatomy, and physiology of the squamate CNS

Amniote brains are organized according to the species-specific development of the highly-divergent telencephalon. Since mammalian and avian brains have highly specialized structures, the evolutionary development of the amniote brain can be determined by comparing the brain structures of prototypic taxa. Owen (1848)

coined the term "homology" to refer to organs that have originated similarly regardless of form and function in different species. Studying homologs allows researchers to deduce the evolution of brain structure and function by determining similar origin.

Brain morphology and neural networks have diverged greatly between vertebrate species in accordance with each clade's unique environment. Several species have developed very specialized brain anatomy and physiology in response to environmental pressures (Butler and Hodos, 2005; Nieuwenhuyis et al., 1998; ten Donkelaar, 1988) while others have simply inherited ancestral traits, thus providing extant evidence for tracing the evolution of morphology (Nomura et al., 2013b). For example, reptiles and birds share several characteristics, including presence of the dorsal ventricular ridge (DVR) and absence of callosal projections. Yet both the structure and function of the avian DVR seem to be entirely different from the reptilian DVR. Understanding the similarities and differences of the DVR not only gives insight into regional function in both of these classes, but it can also give clues as to why the DVR originally formed and how it will continue to develop.

Warner's (1931; 1946; 1947) and Carey's (1967) investigations were the first studies of the snake telencephalon, and have served as the foundation leading to the advent of morphology and pathway studies begun by Ulinski and Halpern in the 1970s (Halpern, 1980; Ulinski, 1974; Ulinski, 1975; Ulinski, 1978). Reptiles are the first vertebrate class in which distinct cortical regions become obvious. They develop physically in a way that is between the amphibian and mammalian telencephalon,

which allows for an intermediate comparison point for making generalizations across vertebrata (Halpern, 1980).

The three clearest distinctions in the reptilian forebrain are the ventricles, the telencephalon, and the diencephalon (fig. 2). The lateral ventricle extends almost completely along the lengthwise aspect of the superior medial telencephalon, bilaterally. It begins as ovoid in shape in the main olfactory bulb, and becomes slit-like and "C shaped" moving caudally into the accessory olfactory bulb and the retrobulbar region. Reptilian brains exhibit a simple, laminated dorsal telencephalon with a 3-layered cortex, expected to be inherited from their amniote ancestry (Bruce, 2007; Wise et al., 2009). Continuing caudally into the midtelencephalon, the ventricle becomes shaped like a "lowercase n" and then a "wishbone." In the mid-caudal regions the septum becomes surrounded by the medial aspect of the ventricle. Further back it makes room for the pineal gland and the medial portion of the ventricle disappears with the septum. The diencephalon and telencephalon separate in the rostral most region as the lateral aspect of the ventricle continues to expand linearly and form a thin circular ependymal border. In this area the lateral ventricle separates the nucleus sphericus (NS) completely from the cortex. Due to the importance of pheromone perception, the NS is very well developed in snakes and occupies almost the entire caudal half of the subcortical telencephalon (Halpern, 1980; Lanuza and Halpern, 1998; Schwenk, 1993).

Squamate olfactory system

Generally, squamates are highly olfactory-dependent as evidenced by their well-developed main olfactory bulbs (Halpern, 1992; Mason, 1992). Predatory, mating, and courtship behaviors are all, in turn, dependent on hypothalamic input in response to these olfactory afferents (Friedman and Crews, 1985a; Friedman and Crews, 1985b). The olfactory system consists of the main olfactory bulb, responsible for capturing odors at the olfactory epthelium and transmitting them to the olfactory bulb, and the accessory olfactory bulb (AOB), responsible for processing pheromones responsible for chemical social communication. These project to the lateral cortex (LC) in reptiles, which is considered homologous to the mammalian olfactory cortex (Hoogland and Vermeulen-Vanderzee, 1995; Martinez-Garcia et al., 1986).

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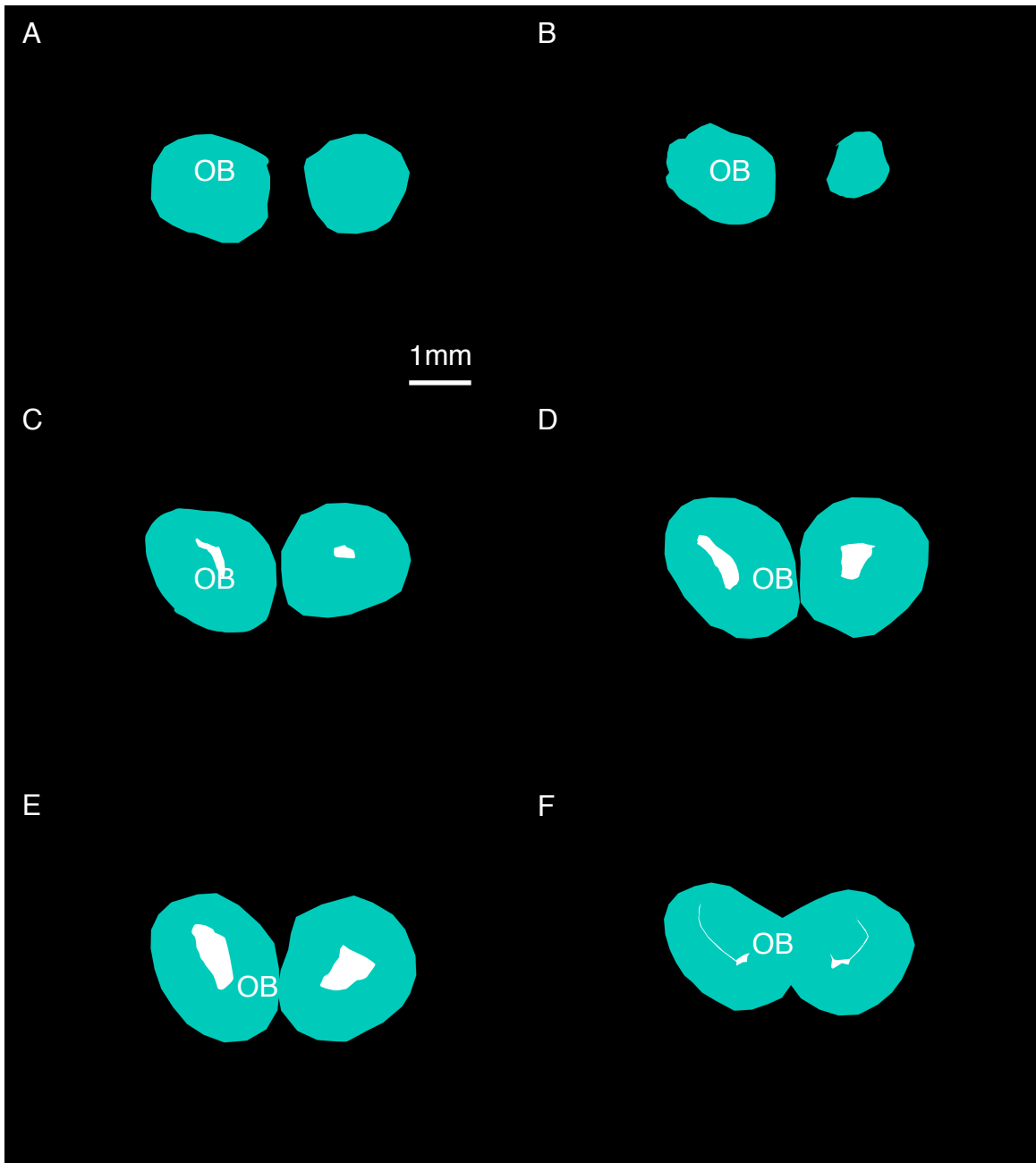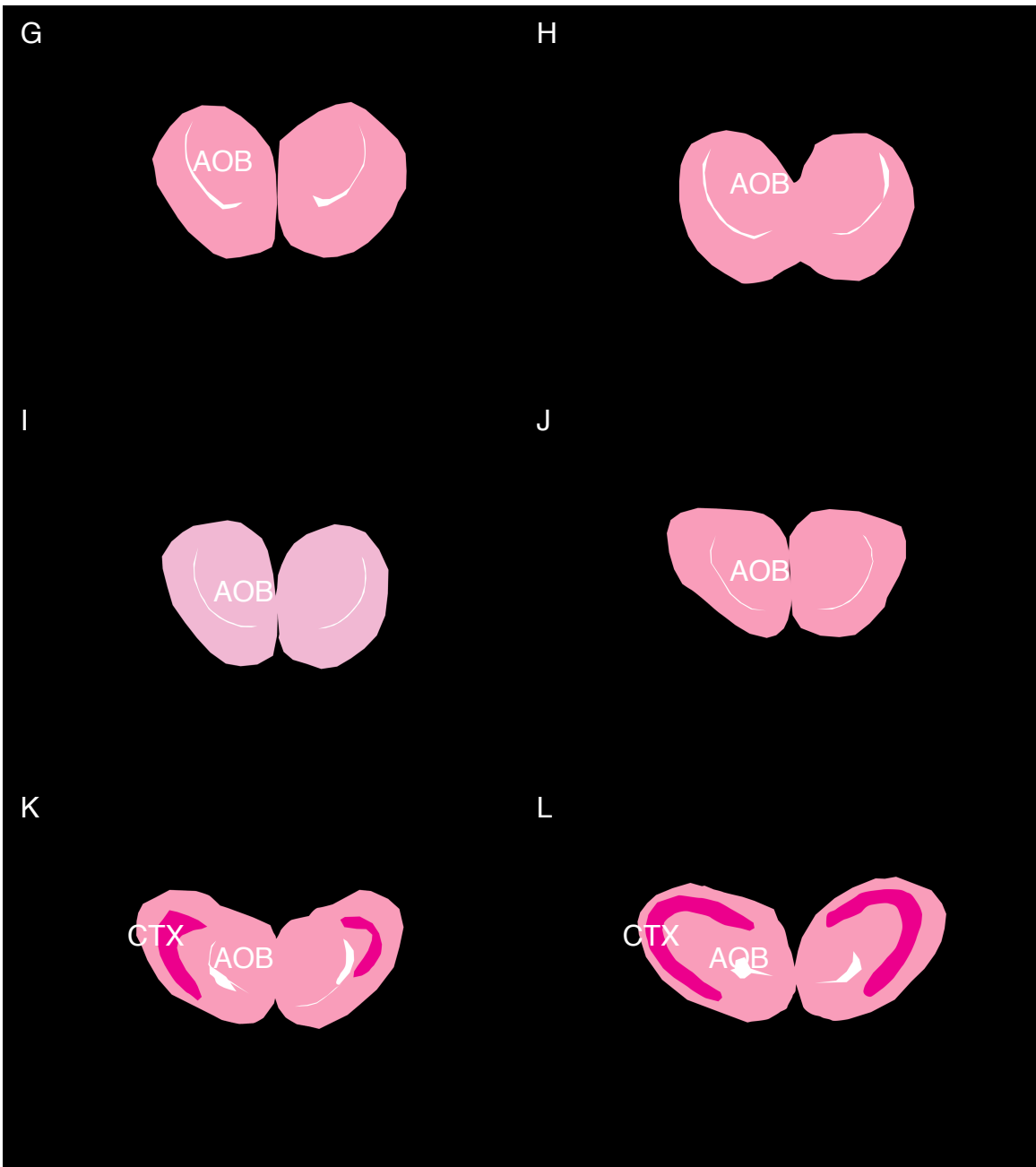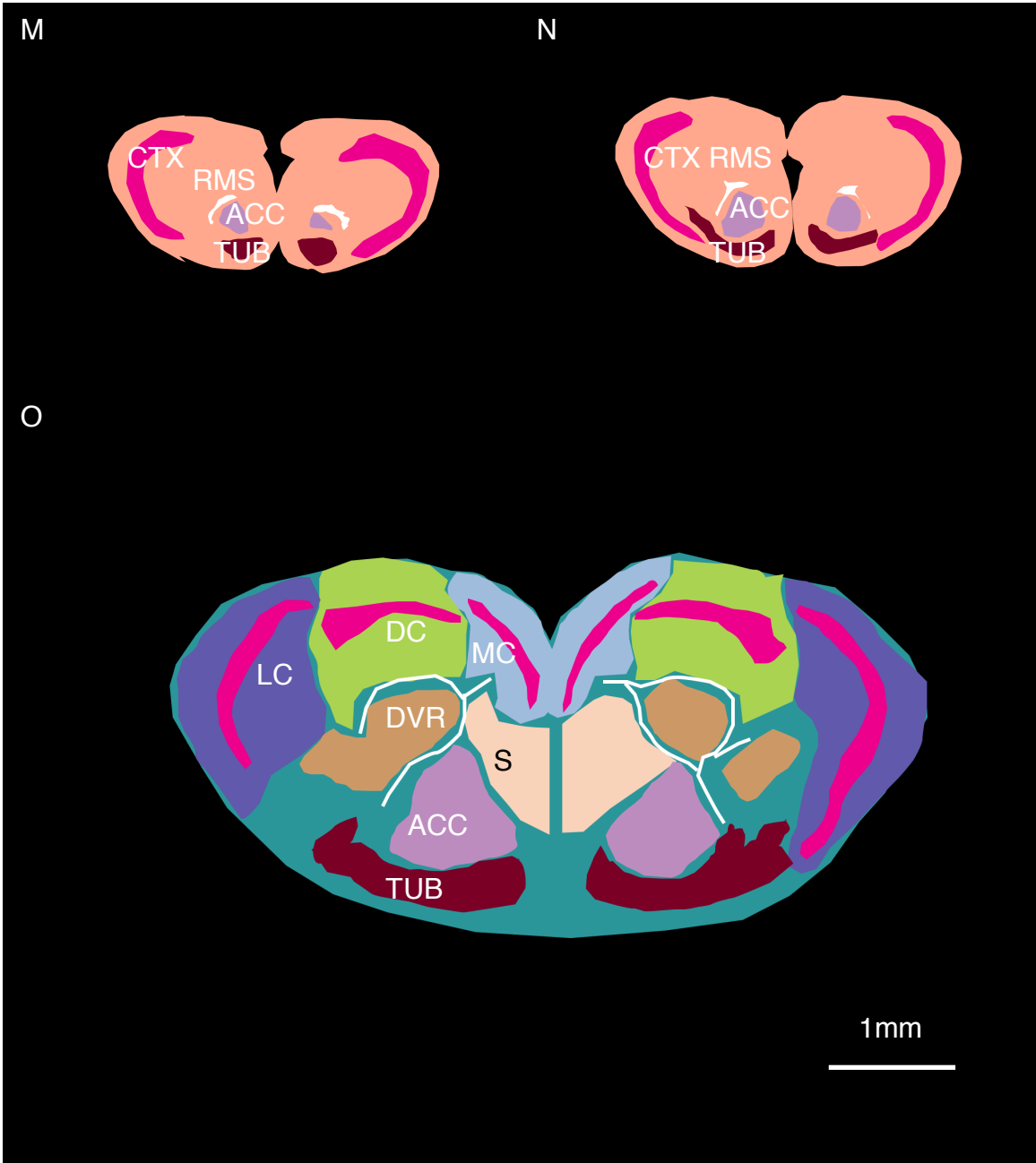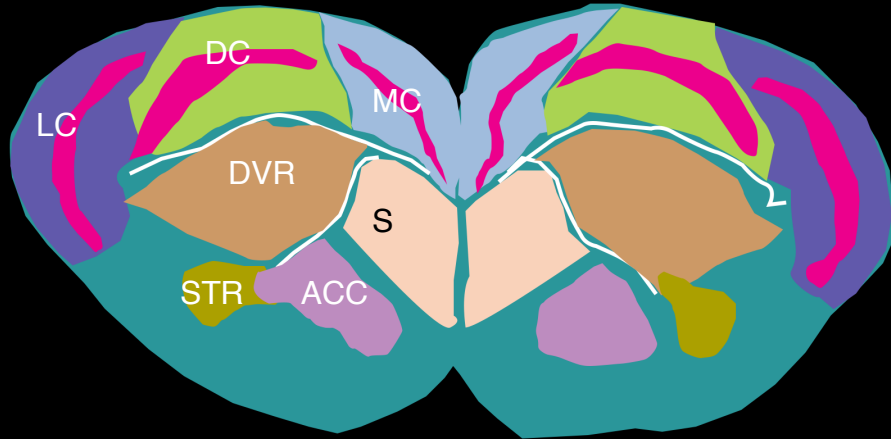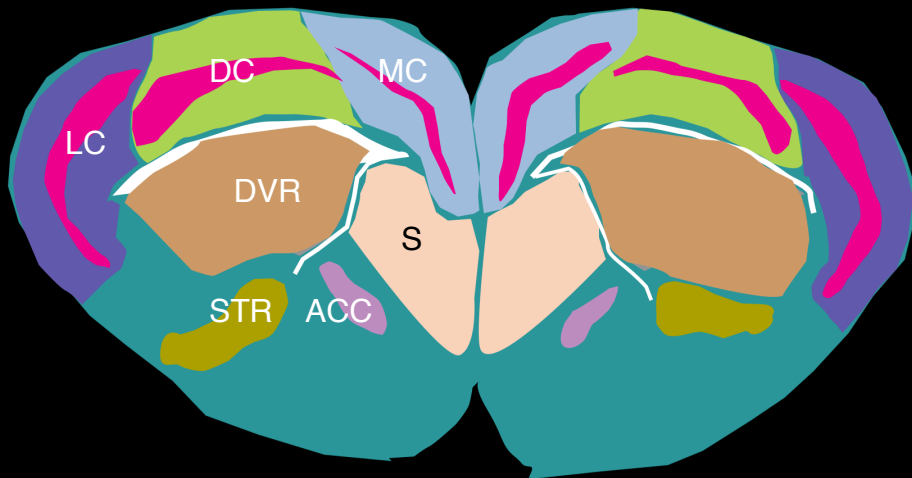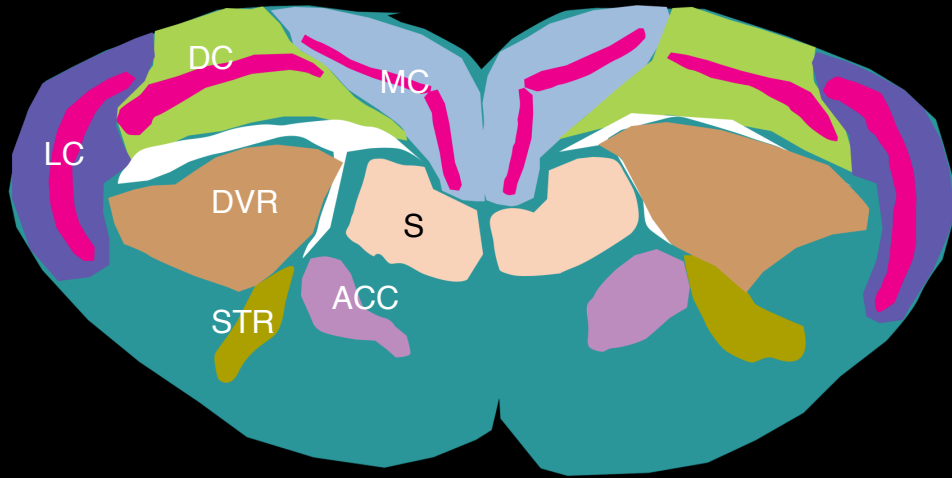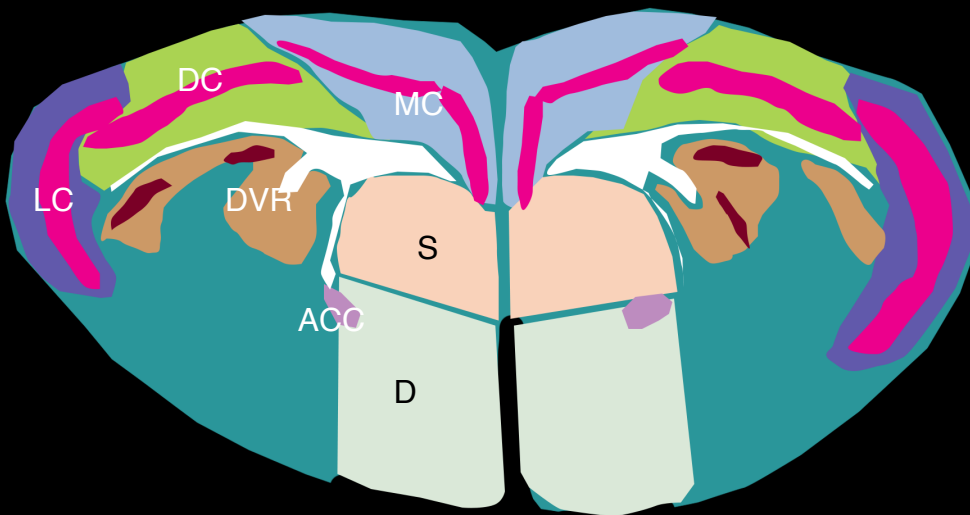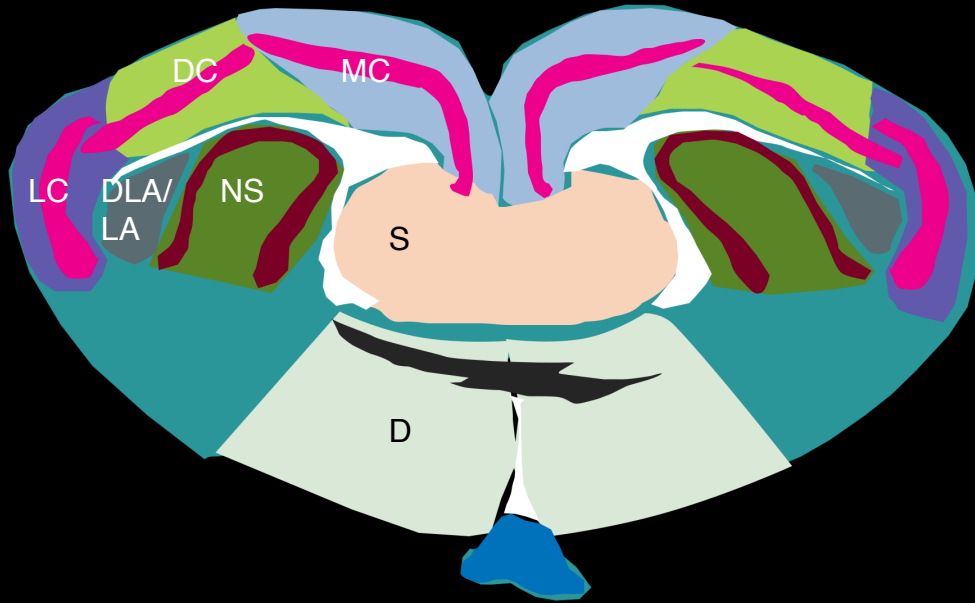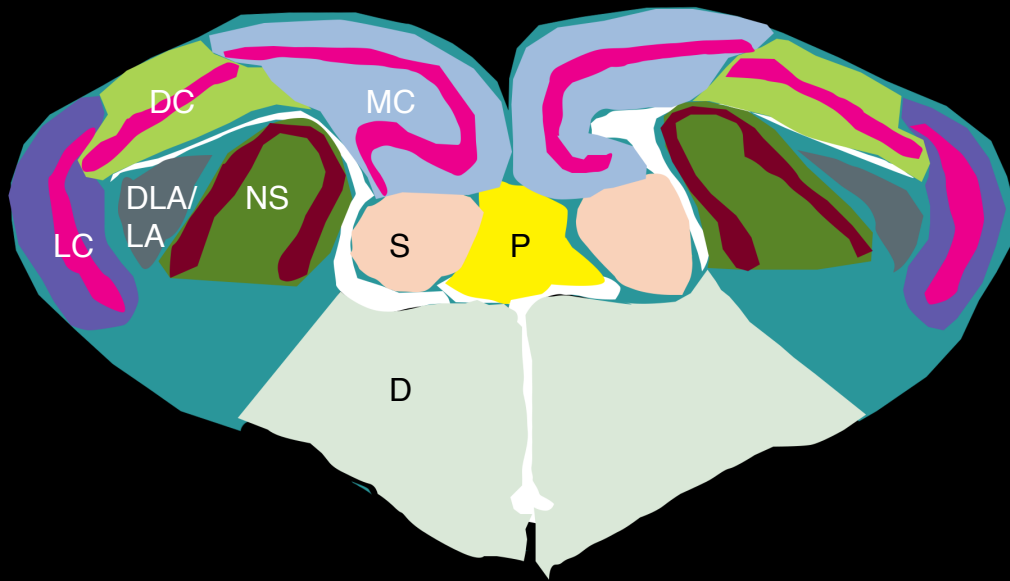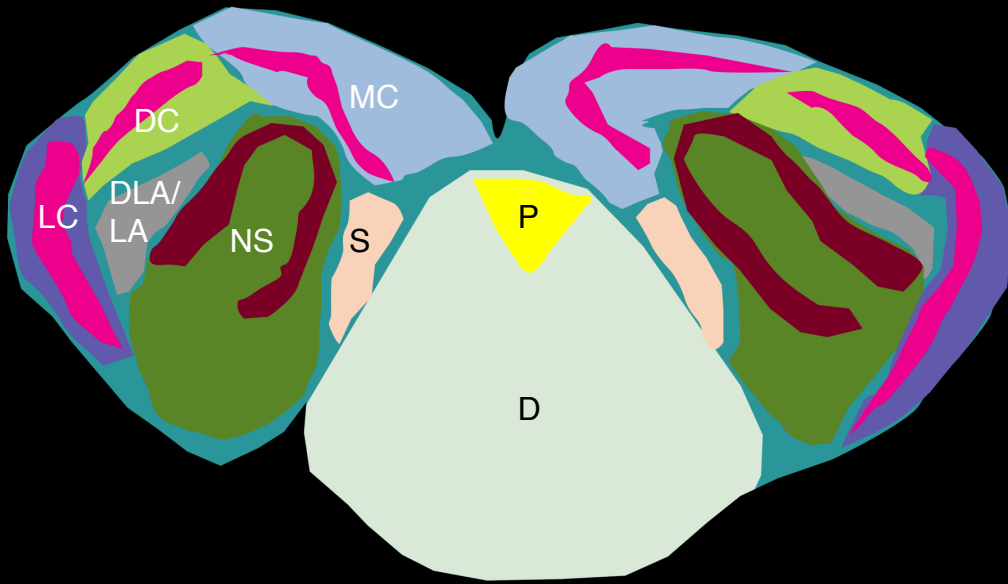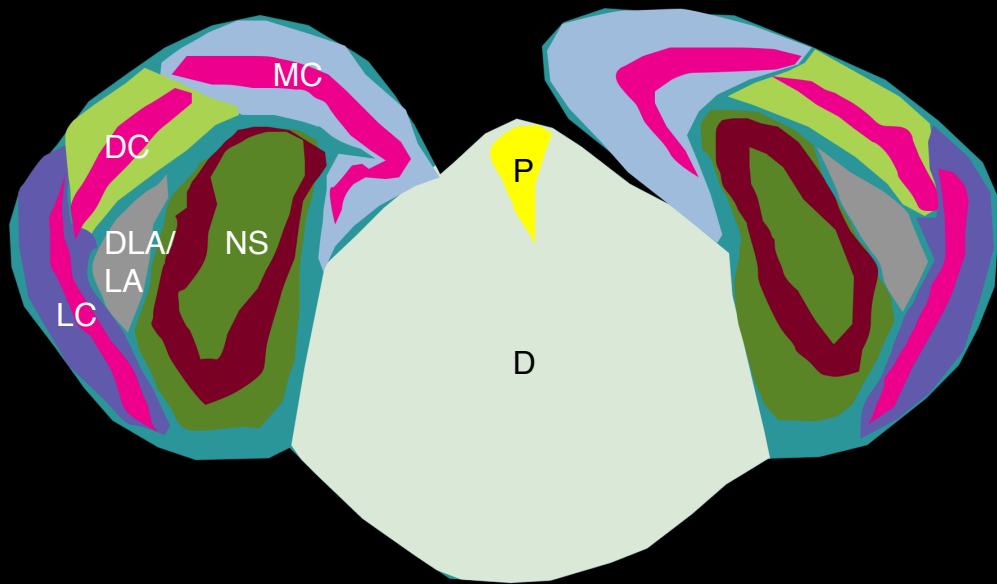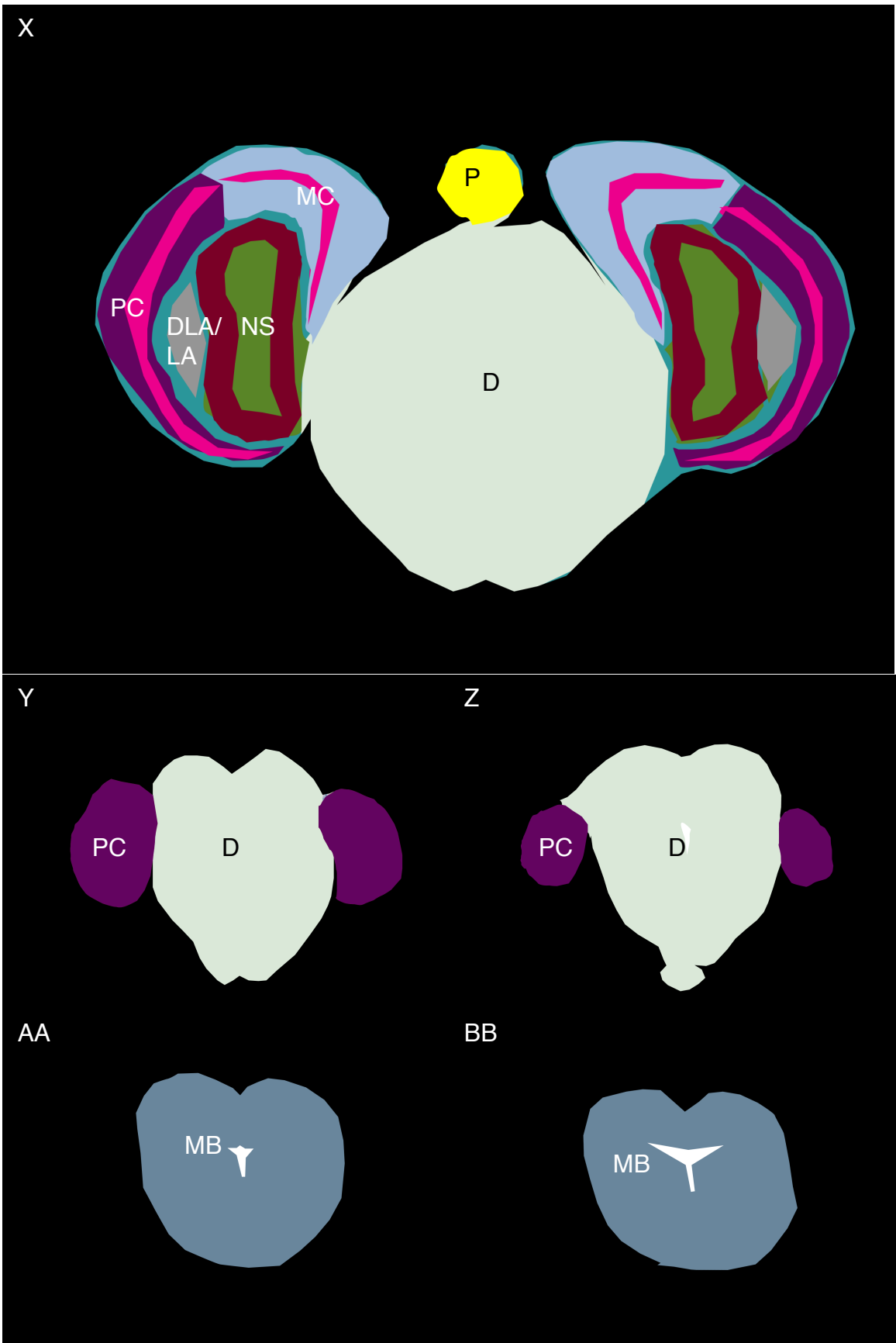
Figure 2 (previous pages): Coronal sections from rostral to caudal through the telencephalon of P. regius highlighting regions determined by comparison of cresyl violet stained sections to Halpern (1980) and Smeets (1988). The telencephalon can be organized into: main olfactory bulb (OB), accessory olfactory bulb (AOB), retrobulbar region, cortical mantle (cortex; Medial Cortex, Dorsal Cortex, Lateral Cortex, Posterior Cortex), septum (S), dorsal ventricular ridge (DVR), the dorsolateral amygdala (DLA) and lateral amygdala (LA), the basal telencephalic structures which are mainly the nucleus accumbens (ACC) in the ventral-medial (VM) region and the striatum (STR) in the ventral-lateral (VL) region, and the nucleus sphericus (NS). Also labeled are the diencephalon (D), pineal gland (P) and midbrain (MB).

The Dorsal Ventricular Ridge

The dorsal part of telencephalon, including both the cortex and DVR, is called the Pallium. Across Reptilia, the most prominent pallial structure is the DVR (Holmgren, 1922; Northcutt, 1981; Ulinski, 1983). Structurally, the DVR is considered the linkage between sources of sensory information and the brain structures that modulate behavior. The anterior portion (ADVR) is considered a polisensorial processing center. Discrete auditory, somatosensory, and visual thalamic pathways terminate in the ADVR, including the infrared pathway from the pit organ. (Bruce and Neary, 1995; Martínez-Garcıa et al., 2009; Striedter, 1997).

Pit organs project to the ipsilateral medullary nucleus of the lateral descending trigeminal tract, which projects to the optic tectum (Kobayashi et al., 1992; Molenaar, 1978b; Newman et al., 1980). Experiments using rattlesnakes have supported that both visual and infared information are then relayed through the nucleus rotundus of the thalamus to the ADVR (collothalamic pathway) (Berson and Hartline, 1988; Schroeder, 1981). Whether the pathway is conserved throughout snakes with pit organs is yet to be determined.

The PDVR on the other hand is an associative center for visual, auditory, somatosensory, and olfactory information. It receives convergent inputs from the thalamus, and direct input from the parabrachial region—most likely visceroceptive, nocioceptive, and/or gustatory signals, and lastly amygdaloid input from the DLA/LA (dorsolateral amygdala and lateral amygdala) (Lanuza et al., 1998; Martínez-Garcıa et al., 2009).

There have been functional studies involving lesioning, stimulus and behavior in reptiles (Distel, 1978; Ivazov, 1983; Sugerman and Demski, 1978; Tarr, 1977), but these studies provide only scarce glimpses into the function of the DVR. Using green iguanas, Distel (1978) found stimulus of the DVR to be associated with only minimal physiological response: Locomotion, head movement, and tongue-flicking were associated with stimulation, however no response was found in approximately 40% of subjects stimulated with the high intensity stimulus, and an even greater proportion elicited no response when stimulated with low intensity. Ivazov (1983) found a decrease in conditioned response when the DVR was lesioned bilaterally in *Pseudopus apodus* (the European legless lizard). The conditioned response was most disturbed by lateral lesioning, which likely affected visual function. Sugerman and Demski (1978) found DVR stimulation elicited defensive responses in *Crotaphytus collaris* (western collared lizards), including defensive display, escape response, and gular extension display. So far there have not been any patterns linking general function of the DVR to stimulus or lesion, and the DVR may in fact be

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more heterogeneous than expected in reptiles, so the lack of data at this point limits any structural-functional hypotheses.

The dominant majority of functional studies of the DVR are in avian species and have shown that the DVR is involved in imprinting, song learning, avoidance learning, tool manufacture, tool use, flexible behaviors, high-order associations, and complex cognition (Rattenborg and Martinez-Gonzalez, 2011). Even these studies, which are considerably greater in number than reptile studies only provide early insights into DVR function. Moreover, functional homology between the avian and reptilian DVR is not clear-cut. While there are many structural similarities, the reptilian pallial thickening, which is outside the reptilian DVR, makes connections more comparable to those of the avian mesopallium than any region inside the DVR (Bruce, 2009).

In studies of birds and reptiles the DVR has been compared to the pallial amygdala and claustrum/endopiriform/piriform amygdalar complexes of mammals (Aboitiz et al., 2003; Bruce and Neary, 1995; González-Granero et al., 2011; Holmgren, 1922; Martínez-Garcıa et al., 2009; Nomura et al., 2013b; Striedter, 1997). This comparison is controversial, however, for some researchers have proposed it is homologous to the lateral (extrastriate and auditory) neocortex, instead (Jarvis et al., 2005; Karten, 1997). Lastly, Molnár and Butler (2002) propose that it is, instead, a combination of both. Molnár and Butler expect the sauropsid ADVR is structurally homologous to the lateral neocotex, basolateral amygdalar complex, and claustrum-endopiriform nucleus because they originate from the same

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collopallial field. Ongoing research is attempting to resolve this discrepancy (Aboitiz, 2011; Butler et al., 2011; Karten, 2013; Luzzati et al., 2009; Martínez-Garcıa et al., 2009; Medina et al., 2013; Medina et al., 2011; Nomura et al., 2008).

DLA and PDVR nomenclature

The DLA and the PDVR have been distinguished in different ways in previous studies. Following Curwen (1939), snake studies labeled the region lateral to the caudalmost NS as "pDVR" (Halpern, 1980; Krohmer and Crews, 1987b; Ulinski and Rainey, 1980). Subsequently, Martinez-Garcia et al. (1991) labeled the region as DLA in lizards, and limited the PDVR to the caudal DVR region without this additional area (Martínez-García et al., 1993). This system has been implemented in many contemporary snake and squamate brain studies (Lanuza and Halpern, 1997b; Lanuza and Halpern, 1998; Martínez-García et al., 2007). Simultaneously, several snake brain studies have continued to use the previous system (Holding et al., 2012; Krohmer et al., 2010; Krohmer et al., 2011). A dialogue should occur between researchers to further consistency in future studies. I will use DLA/LA combined, separately from the PDVR, for labeling this region in its entirety. The medial amygdala was not labeled as a region for this study because it could not be discerned using cresyl violet stain.

The amygdala

In reptiles, the amygdala is a general term used to refer to amygdalo-hypothalamic circuits that (a.) relay chemosensory input to the hypothalamus to influence chemically-guided behaviors (Martínez-Marcos et al., 1999) and (b.)

receive inputs from sensory and associative areas (Martínez-García et al., 2007). It

is not a monolithic structure, but instead a collection of nuclei—a somewhat arbitrary

assemblage that is "neither a structural nor functional unit" (Swanson and Petrovich,

1998). The amygdala of the common garter snake, *Thamnophis sirtalis*, has been

shown to be part of a complex system, in which the following amygdalo-

hypothalamic projections occur: (a) The olfactory amygdala receives input from the

NS and then projects back to the NS, PDVR, and hypothalamus; (b) the NS projects

directly to the hypothalamus; (c.) the medial amygdala receives input from the LC

and NS and transmits to the hypothalamus; (d.) the PDVR receives afferents from

the LC and amygdala and projects to the hypothalamus; (e.) the DLA receives inputs

from the LC and NS to project to the hypothalamus (Martínez-Marcos et al., 1999);

and (f.) the medial amygdaloid nucleus and the NS are efferents of the accessory

olfactory bulb (Bruce and Dear, 1995a, 1995b).

When making comparisons to the mammalian amygdala, the comparable

reptilian structures are yet to be clarified. This occurs partly because the mammalian

amygdala is still being characterized using developmental, neurochemical, and

hodological (interconnection and pathway-based) approaches (Martínez-García et

al., 2007). The reptilian LC may be homologous to the mammalian posterior lateral

cortical amygdala, and the NS might be homologous to the posterior medial cortical

amygdala (Eisthen and Polese, 2007; Martínez-Garcıa et al., 2009). As previously

mentioned, the PDVR not included in the DLA has been considered a homolog of the

mammalian pallial amygdala, but this comparison is still awaiting clarification.

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The DLA is the main link between the striatum (STR) and the amygdalar

pathways and is likely the reptilian homologue to the basal nuclei of the mammalian

amygdala (Bruce, 2009; Martínez-García et al., 2009). The mammalian basal nuclei

seem to have an essential function in the acquisition and expression of fear

conditioning (Amano et al., 2011). While comparisons have been attempted in birds

(Bruce, 2009), the lack of a vomeronasal system, and poorly developed olfactory

system has considerably limited the accuracy of comparison (Moreno and González,

2007). In anurans, three main amygdaloid subdivisions have been discovered, the

lateral amygdala, medial amygdala, and central amygdala. Similar to the basal nuclei

of mammals, the anuran lateral amygdala is responsible for association and labeling

of chemical odors, pheromones and non-chemical visual, auditory, somatosensory,

stimuli. It provides emotional labeling of stimuli, creating an "emotive memory,"

allowing for conditioning of stimuli (Moreno and González, 2007).

The dorsal and medial cortex

The reptilian cortex is comprised of three-layered laminar structures that may

have been acquired by early amniotes (Wise et al., 2009). The cortex consists of a

cell-dense central layer surrounded by cell-sparse outer and inner plexiform layers

(Ulinski, 1990). The ophidian cerebral cortex is divided into three primary regions,

the medial, dorsal, lateral, and posterior cortices (MC, DC, LC), and a fourth sub-

region, the dorsomedial cortex. The dorsomedial cortex, located between the medial

and dorsal cortex, was first described as a separate subregion as early as 1917, and

was originally named "zona piramidial curvilinear" by Ramon y Cajal (1917); (1918).

It has only been found in squamates and it does not develop independently until after birth (Lopez-Garcia et al., 1984). For this study it was difficult to distinguish dorsomedial cortex from the medial cortex so only the three main regions are used. They all extend longitudinally from the anterior olfactory bulb (rostralmost) to a fifth region, the posterior cortex (PC; caudalmost) of the telencephalon. I have noted the PC as a separate part of the cortex because it is difficult to distinguish different cell layers in the hindmost portion of the cortex, and while it is most likely the caudal continuation of the MC, cells could also originate from the DC and LC layers.

In mammals, the hippocampus is considered a center for spatial memory. The reptilian MC and DC are generally regarded as homologous to the mammalian and avian hippocampus (Bruce and Butler, 1984; Butler and Hodos, 2005; Rodriguez et al., 2002). In reptiles, spatial learning has been demonstrated in *Elaphe guttata guttata* (corn snakes) (Holtzman et al., 1999) and *Tiliqua rugosa* (blue tongued skinks) (Zuri and Bull, 2000). Also, lesioning the medial and dorsal cortices of turtles causes a reduction in maze navigation ability (Lopez et al., 2003; Rodriguez et al., 2002) and produces deficits in a variety of non-spatial tasks such as discrimination and restraint habituation (Blau and Powers, 1989; Grisham and Powers, 1989; Moran et al., 1998). In squamate reptiles including snakes, the MC is also associated with navigation ability (Baird Day et al., 1999a; Baird Day et al., 1999b; Crews and Wilczynski, 2000; Holding et al., 2012; LaDage et al., 2009; Roth et al., 2006).

Similarities also exist in the development and growth of the reptilian MC and mammalian hippocampus. Neuroblasts of the reptilian MC express PSA-NCAM during migration similarly to the neuroblasts of the mammalian hippocampus (Ramirez-Castillejo et al., 2002b; Seki and Arai, 1993). Also similar to the hippocampus in birds, the two neuronal types that make up the MC in lizards are small (type I) and large (type II) neurons (Kirn et al., 1999; Lopez-Garcia et al., 1984). As lizards age the neurons in the MC convert from large to small, possibly due to the chromatin condensation process. This transformation either is species specific or has not been explored at enough time points in enough species to capture its universality (Pérez-Cañellas and García-Verdugo, 1996).

Nevertheless, there is some question as to whether the MC is indeed homologous to the hippocampus. Baird Day et al. (2001) argued that perhaps only navigation, and not spatial memory are altered by lesioning. When challenging *Cnemidophorus inornatus* (little striped whiptail lizards) to locate a heated rock in a circular arena they found lesioning the DC and MC slowed the lizards' ability to locate objects, and altered their search strategies. Still, none of the lizards in the cortex-lesioned or sham-lesioned groups adopted a spatial strategy for locating objects, indicating that lizards may not be utilizing true "spatial memory" per se. Instead the MC may be necessary for flexible navigational learning, and the DC may be involved in remembering unchanging external cues. This has been supported by Jacobs (2003) who expects lizards are orienting according to directional cues like magnetic fields, temperature, color, auditory or light cues, and not with true spatial

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memory. Jacobs also gives two potential explanations for the discrepancies between turtles and lizards: (a) turtles are relying on a bearing map instead of a sketch map, or (b) turtles visual cognition is functionally and anatomically different from lizards (Bruce and Butler, 1984; Hall and Ebner, 1970). This is actively a point of contention among researchers and is still undergoing investigation (LaDage et al., 2012).

The retrobulbar regions

Rostral to the cortices, the retrobulbar region is the region directly caudal to the AOB. It is where the dorsal and lateral pallium forms, and becomes semicircular on the dorsal surface posteriorly, forming the rostralmost cortex. The cortex is not yet distinguishable as three different cell layers (MC, DC, LC) and instead forms a single cell layer. The pallium rapidly enlarges caudally, laterally and dorsomedially, to comprise the majority of the dorsal telencephalon.

The largest nucleus of the retrobulbar anterior ventral (AV) region is the olfactory tubercle (TUB). The TUB links directly to the olfactory bulb, to relay and processes olfactory signals. In reptiles there has been very little study of the TUB, other than some afferent and efferent tract tracing studies (Halpern, 1976; Lanuza and Halpern, 1997a; Lanuza and Halpern, 1998). Yet the TUB has been considerably studied in mammals, and these studies have uncovered that it receives afferents from multiple sensory, storage, neuroendocrine and limbic structures (Budinger et al., 2006; Fallon, 1983; Groenewegen et al., 1987; Mick et al., 1993; Scott et al., 1980; Ubeda-Bañon et al., 2008; Vertes and Hoover, 2008). This

aggregation of so many systems at the mammalian TUB indicates that it may

actually be an integrator of multimodal information (Wesson and Wilson, 2010).

Functional studies in humans have shown the TUB is essential to the

discrimination of the source (type) of olfactory information (Zelano et al., 2007), and

the direction of attention to smells (Zelano et al., 2005). In other mammal studies,

the TUB also plays a role in behavior (Hitt et al., 1973). Bilateral lesioning has been

found to reduce copulation in male rats (Hitt et al., 1973). Lesioning has also been

found to cause hyperphagia and attenuates increases in locomotion associated with

dextroamphetamine. (Koob et al., 1978). The most studied aspect of the TUB is its

role in addiction and reward systems (Heimer, 2003; Ikemoto, 2007). Ikemoto (2003)

found that when rats are able to self-administer intracerebral infusions of cocaine to

several brain regions, the olfactory tubercle is more rewarding than the ACC, and

successfully elicits conditioned place preference. It is likely the TUB plays an

essential role in odor-motivated behavior, including approach/avoidance,

conditioning, and learning. Given our lack of information on the function of the TUB

in non-mammalian vertebrates, there is a whole area of comparison to non-

mammalian species open for discovery.

The basal forebrain

In the ventral side of the retrobulbar telencephalon, the striatum (STR) and

olfactostriatum form (Durward, 1930). The nucleus accumbens (ACC) lies between

the septum and paleostriatum (Warner, 1947), and may be considered part of the

paleostriatum (Halpern, 1980). Compared to other reptiles, the python has a very

large ACC and a small STR (personal observation), however no researchers have investigated functional or evolutionary aspects of this distinction (Smeets, 1988).

The ACC is the dominant structure of the VM portion of the *P. regius* telencephalon. In mammals, it has been implicated as responsible for motivation and reward-seeking behavior (Cador et al., 1989; Knutson et al., 2001), to the point where rats will self-administer amphetamine directly to the ACC in a feedback loop of addiction (Hoebel et al., 1983). Furthermore, the ACC is essential to the flexible facilitation of rewards and the avoidance of negative stimuli, and the construction of learned incentive structures (Ikemoto and Panksepp, 1999). The mammalian ACC is structurally, hodologicallly, neurochemically, and developmentally similar to the reptilian ACC (Gonzalez et al., 1990; Guirado et al., 1999; Russchen and Jonker, 1988; Smeets and Medina, 1995; Smeets, 1988; Smeets et al., 1986; Smeets et al., 1987). When male *Eublepharis macularius* (leopard geckos) hatch from eggs incubated at female-biased temperatures, they maintain higher dopamine (DA) levels in the ACC when encountering females, compared to those hatched at male-biased temperatures (Dias et al., 2007). Since males hatched at female-biased temperatures are more sexually active (Flores et al., 1994; Sakata and Crews, 2003) and exhibit greater anticipatory behavior when exposed to females (Sakata and Crews, 2003), these findings may serve as a jumping-off point for studying the function of the ACC and its influence on behavior in reptiles.

The STR embodies the majority of the VL region. In mammals, it is the key nucleus of the basal ganglia, involved in the selection of non-adverse (Darvas et al.,

2011; Dombrowski et al., 2013) and pro-rewarding actions (Da Cunha et al., 2012; Kravitz et al., 2012; Yin et al., 2004). It is densely innervated with dopaminergic neurons from the midbrain nigrostriatal pathway, and its dysfunction is implicated as responsible for hypokinesia (e.g. Parkinson's disease) (Li et al., 2009) or hyperkinesia (e.g. chorea, dystonia) (Mink, 2003).

Currently, the mammalian, avian, and reptilian STR all appear to be hodologically, neurochemically, and developmentally similar (Gonzalez et al., 1990; Moreno et al., 2010; Russchen and Jonker, 1988; Smeets, 1988; Smeets et al., 1986; Smeets et al., 1987). Lesioning the reptilian STR in male *A. carolinensis* causes a reduction in male-typical assertation display (Greenberg, 1977). Stimulation, on the other hand, elicits assertation display, challenge display, and locomotor responses in *S. occidentalis* (Tarr, 1982).

The septum

The septum is the region medial to the lateral ventricle. Carey (1967) determined that there are eight septal nuclei in *Coluber constrictor constrictor* (Blacksnakes). Crosby et al. (1967) determined that there are nine nuclei in *C. constrictor constrictor*. Some clarification has also been found in other reptiles (Font et al., 1995a; Font et al., 1998a; Font et al., 1998b; Font et al., 1997a). In comparative studies, there are three major divisions to the septum (lateral, medial, and caudal), which all tetrapod vertebrates share (Lanuza and Martínez-García, 2009). Lanuza and Martínez-García (2009) argue the similarities of these divisions can serve as evidence of the essential and conserved functions of the septum.

Studies in reptiles confirm some homology with the mammalian septum, and have revealed: (a.) The lateral septal complex receives excitatory input from the hippocampal cortex and generates inhibitory efferents to the medial ventral pallidum and motor parts of the hypothalamus; (b.) the medial septal complex sends inhibitory signals to the hypothalamus; and (c.) the caudal division (triangular nucleus) projects mainly to the habenula (Font et al., 1998b; Swanson and Risold, 2000).

Font et al. (1998b) posited that the lateral septum is involved in the expression of appetitive behaviors (e.g. food and water intake), aggressive/defensive behavior, and reproductive physiology in reptiles, and may be considered a "distributor of behaviors" (Distel, 1978; Krohmer and Crews, 1987a; Tarr, 1977). Concurring with functional rat studies, the septum may mediate social memory including olfactory cues, parental behavior, intraspecific aggression, dominant-subordinate relationships, defensive/agonistic behaviors and territoriality; which is why it is considered key to the social behavior network (Dantzer et al., 1988; Lanuza and Martínez-García, 2009; Numan, 2000; Sheehan and Numan, 2000; Yang and Wilczynski, 2007). The main afferent to the lateral septum is a projection from the hippocampal formation, which provides spatial and non-spatial contextual information in mammals (Sheehan et al., 2004). Hence the lateral and medial septum are possibly responsible for initiating territorial behaviors in reptiles, like in mammals, in response to contextual cues (Newman, 1999).

6. History of adult neurogenesis research

Over the past 50 years, the study of adult neurogenesis has developed from marginal origins to become an integral part of neuroscience research. For most of the 20th century, neurogenesis (the process of generating new neurons from progenitor cells) was assumed to occur only in prenatal and early developmental stages of organisms (Gross, 2000; Ramón y Cajal, 1928). The proliferation of neurons in adult mammals was first discovered by Altman (1962) but received little attention. It was the development of techniques like 5-bromo-2'deoxyuridine (BrdU) immunohistochemistry (IHC) and confocal microscopy in the 1980s and 1990s that convinced researchers that adult neurogenesis indeed occurs across a wide range of taxa. The discovery of neurogenesis in birds during the 1980s prompted researchers to look at mammals again (Goldman and Nottebohm, 1983). In 2000, Gross declared that the long-standing dogma that new neurons cannot be added to the adult brain had been substantially overturned.

While the advent of biotechnology has generated considerable interest in neurogenesis, researchers have not yet elucidated the mechanisms of adult neurogenesis or how it impacts behavior, particularly in non-mammalian species. Several investigators have emphasized the importance of studying adult neurogenesis in a wide range of species, in both laboratory and natural environments (Font et al., 2001; Lindsey and Tropepe, 2006). Others have proposed that organisms need to be subjected to various experimental manipulations in order to discern what factors affect neurogenesis (Kaslin et al., 2008; Kempermann et al.,

1997). In rats, for example, learning tasks, larger cages and novel objects reduce the death rate in newly born neurons (Gould et al., 1999; Kempermann et al., 1998). Further, in mammals, sleep and exercise both positively affect neurogenesis (Meerlo et al., 2009; Olson et al., 2006; van Praag et al., 1999) and stress negatively affects neurogenesis (Gould et al., 1997; Gould et al., 1998). Powers and Hanusch (2012) have begun to investigate the effect of enriched environments on neurogenesis in reptiles using painted turtles (*Chrysemys picta*).

7. Use of markers to study neurogenesis

Tritiated thymidine autoradiography is a technique used to study cell proliferation, migration and differentiation. The injected tritiated thymidine is incorporated into the DNA during S-phase of the cell cycle marking only new cells during their mitosis. After the organism is sacrificed and the tissue is fixed, sections are observed by autoradiography, which provides a snapshot of new cells undergoing dynamic processes of birth and development. While this method marks new cells very effectively for observation and quantification under microscopy, tritiated molecules emit beta radiation, and can be absorbed through the air or the skin, and remain in the body as a long-term carcinogen.

In the 1980s BrdU was developed as a marker for proliferating cells (Gratzner, 1982; Miller and Nowakowski, 1988). BrdU has subsequently become the preferred neurogenesis marker in developing and adult brains because it utilizes immunohistochemistry (IHC) instead of autoradiography (Abrous et al., 2005; Christie and Cameron, 2006; Kuhn and Cooper-Kuhn, 2007; Wojtowicz and Kee,

2006). Like tritiated thymidine, BrdU is a thymidine analog that incorporates into DNA during S-phase. However instead of potentially dangerous radiolabelled molecules, BrdU uses brominated molecules. Anti-BrdU antibodies can then bind to the brominated region and be bound by biotinylated or fluorescent antibodies for observation.

While early studies used 50mg/kg doses of BrdU, Cameron and Mckay (2001) and Gould and Gross (2002) determined that increasing the dose of BrdU injected in rats to 300mg/kg labeled significantly more new cells at the end of 24 hour and 4 week periods. In contrast, Burns and Kuan (2005) double labeled with Iododeoxyuridine (IdU) and found that the standard doses of 50mg/kg-100mg/kg labeled only ~10% fewer cells than the 300mg/kg dose in mice. Hancock et al. (2009) also found significant differences between the BrdU doses of 40mg/kg and 240mg/kg, but did not find differences between 240mg/kg and 480mg/kg doses, supporting Gould & Gross's original finding and implying the optimum dose in rats may in fact be between 200mg/kg and 300mg/kg. So far all dosage studies have been performed in mammals and differences in the effect of dosage are yet to be determined in reptiles and birds.

When administering BrdU, there is a distinct difference between embryonic and postnatal brains in regard to toxic effects. Bannigan (1985) found that BrdU-labeling induced cell death in embryonic mouse brain cells. Cameron and Mckay (2001) found that adult rat brains, on the other hand, are not harmed. This result was

also supported by Hancock et al. (2009), who did not find damage to the rat hippocampus after BrdU administration.

Inconsistency among previous BrdU-based adult neurogenesis experiments has prevented researchers from being able to make direct comparisons. Quantification practices are inconsistent among experiments due to differences in dosages and antibodies used for detection. For example, (Leuner et al., 2009) found that Dako, Roche, BD, and Accurate brand antibodies against BrdU labeled more rat hippocampal cells than those from Vector and Novocastra.

In mammals, the number of cells labeled with a single injection of BrdU doubles between 2 hours and 24 hours after injection. This indicates that BrdU must be labeling only dividing cells. A concern has been whether or not BrdU is not just integrated into new cells, but also cells undergoing DNA repair. In studies of the normal mammalian brain, DNA repair is constant (Korr et al., 1989; Korr and Schultze, 1989; Schmitz et al., 1999). DNA repair has only been marked by BrdU in studies where cultured non-neuronal cells are damaged by genotoxic chemicals or UV exposure (Selden et al., 1994; Selden et al., 1993). Schmitz et al. (1999) found that no studies have identified BrdU labeling of DNA repair (*in vivo*) in mice. Molecularly, the anti-BrdU antibody clone commonly used (IU-4) requires divalent antigen binding, which is not likely to occur during in vivo repair (Selden et al., 1994).

The use of subcutaneous injections may also influence the required dose of BrdU necessary for studying adult neurogenesis in snakes because of the permeability of the blood brain barrier to nucleosides. Previous studies of

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neurogenesis in mammals support the likelihood that the blood-brain barrier restricts thymidine entry into the brain (Bradbury, 1979; Das and Altman, 1971; Kaplan, 1982). BrdU is transported by active and facilitative nucleoside transport systems comparable to thymidine (Lynch et al., 1977). However, no studies have been performed comparing injections into the peritoneum (intraperitoneal; IP) or subcutaneous injections to direct injections in the cerebral ventricles (intracerebroventricular; ICV) in snakes or other reptiles. Thus the permeability of the blood-brain barrier to thymidine or BrdU and the density of nucleoside transporters are yet to be established.

To determine whether ^{3}H-Thymidine- or BrdU-marked cells are neurons, people have used a variety of techniques to demonstrate anatomically that these cells are neurons, such as electron microscopy to show the presence of dendrites and synapses (Lopez-Garcia et al., 1988) or light microscopy to identify the large nucleus and prominent nucleoli of neurons (Goldman and Nottebohm, 1983). Possibly the most accurate and convenient way to determine the phenotype of different brain cell types is to use antibody markers such as doublecortin (DCX), glial fibrillary acidic protein (GFAP), hexaribonucleotide binding protein-3 (Fox-3/NeuN), protein gene product 9.5 (PGP 9.5) and III- β tubulin (Tuj1). For neurogenesis studies, antibodies for these neuronal or glial markers can be paired with anti-BrdU antibodies to perform double- or triple-immunofluorescence. The cells can then be observed with fluorescence or confocal microscopy to determine whether new cells are neurons or glia. Generalized surveys quantifying adult neurogenesis using

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confocal methods have been very limited in reptiles and no studies have used these techniques for surveys in snakes (Delgado-Gonzalez et al., 2011; Pérez-Cañellas and García-Verdugo, 1996; Sampedro et al., 2008; Shao et al., 2012).

8. Comparative studies of adult neurogenesis; Adult neurogenesis in reptiles

Adult neurogenesis is widespread and varies both among and within taxa (Barker et al., 2011). In order to make generalizations about the nature of adult neurogenesis, further studies of a wide range of taxa will allow for further clarification of the trait's evolutionary development. Relying on a few models prevents the delineation of evolutionary trends (Lindsey and Tropepe, 2006). Further, comparison between several members of the same class allows for the determination of consistency of structure and function within the given class (Rakic, 2002). For example, adult neurogenesis has not been found in adult Sorex shrews (Bartkowska et al., 2008) or the hippocampus of some bat species (Amrein et al., 2007), yet it is present in the six other mammalian orders investigated thus far. Understanding which evolutionary pressures have distinguished some vertebrates as better or worse at adult neurogenesis might help clarify the functions and benefits of this physiological process.

Neurogenesis is very limited in the adult mammalian brain. Reptiles, in contrast, are model examples of extensive adult neurogenesis in vertebrates. While birds are included in the monophyletic reptilian group, "reptiles," for the purposes of this paper, I will refer to reptiles without including birds. All reptiles examined to this

point exhibit adult neurogenesis. Yet there are considerable differences in the rate and distribution of adult neurogenesis among species within this group (Font et al., 2001).

As early as the 1950s, researchers claimed that neurogenesis might occur in adult reptiles along the ependymal lining of the ventricles (Fleischhauer, 1957; Källen, 1951; Kirsche, 1967; Schultz, 1969). Subsequently it was assumed that these regions were merely embryonic remnants that persisted into adulthood. In the 1980s and 1990s it was determined that new adult brain cells proliferate at the ventricular zone (VZ), the lining of the ventricles, and migrate away to their final destination in the parenchyma (Goffinet, 1983; Lopez-Garcia et al., 1988; Lopez-Garcia et al., 1990; Pérez-Cañellas and García-Verdugo, 1996).

Proliferation

The production of new cells in birds and reptiles is restricted primarily to the ependymal VZ of the lateral ventricle (Alvarez-Buylla et al., 1990; Lopez-Garcia et al., 1988; Lopez-Garcia et al., 1990). Reptilian ependymal cells lining the ventricular wall proliferate continuously, generating new neurons that integrate into established circuits (Lopez-Garcia et al., 2002; Marchioro et al., 2005). The ependymal regions associated with highest proliferative capacity are referred to as the sulcus lateralis, sulcus septomedialis, sulcus ventralis, and sulcus terminalis located along the lateral ventricles (Kirsche, 1967; Schultz, 1969; Tineo et al., 1987; Yanes-Méndez et al., 1988) The third, tectal and fourth ventricles may also be a source of new neurons and should be investigated further (personal observation; Margotta et al., 1999;

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Saijo, 2007). In lizards, the neurogenic regions are composed of two main cell types.

Most are radial glial cells while the rest are young migrating neurons (García-

Verdugo et al., 1981; Yanes et al., 1990; Yanes-Méndez et al., 1988; Yanes-Mendez

et al., 1988). Radial glial cells in the VZ are likely the stem cells for adult

neurogenesis in reptiles but evidence supporting this hypothesis in reptile studies is

limited (Font et al., 1995b).

Migration

All vertebrates studied thus far have exhibited cells which migrate tangentially

away from the ventricular wall of the telencephalon (Doetsch and Scharff,

2001).While these new cells migrate throughout the telencephalon, the olfactory

bulbs, and the AOB, there are differences in how they incorporate across species

(Font et al., 2001). The morphology of migrating neurons in the Western Canaries

lizard (*Gallotia galloti*) changes during migration in a way that indicates maturation

en-route. Near the ependymal (VZ) layer the cell nucleus is 3-4 μm in diameter and

elongated in shape with a visible centralized nucleolus and spongy chromatin.

During migration, the chromatin becomes clumped and the nucleus increases in

volume by up to 35% (Garcia-Verdugo et al., 1986). New neurons in the MC mature

and extend axons to their targets upon their arrival at their destination and are

incorporated into pre-existing functional circuits (Lopez-Garcia et al., 1990; Ulinski,

1990).

Survival

Newly generated neurons appear to have long survival at their targets in the telencephalon of lizards (Pérez-Cañellas et al., 1997). While neurogenesis coincides with neuron death in birds (Barnea and Nottebohm, 1994; Barnea and Nottebohm, 1996) and mammals (Hastings and Gould, 2003), no studies have directly linked naturally occurring neuronal degeneration or apoptosis to neurogenesis in reptiles (Barker et al., 2011; Lopez-Garcia et al., 1990).

The MC is continuously neurogenic throughout the life of reptiles (Bruce and Butler, 1984; Butler and Hodos, 1996; Casini et al., 1986; Hoogland et al., 1987, 1998; Lopez-Garcia et al., 1992, 2002), yet the rate of maturation appears to be age-dependent. Molowny et al. (1995) observed more reactive neurogenesis in the MC of young adult Iberian wall lizards (*Podarcis hispanica*) compared to older counterparts. Furthermore, cell maturation in a 2-4 year old *P. hispanica* takes only 7 days (Lopez-Garcia et al., 1990), but takes 90 days in 6-year-old *Gallotia galloti* (Delgado-Gonzalez et al. 2011)*.*

General Comparisons

Interspecific regional differences in neurogenesis are evident among reptiles. For example, the new neuron incorporation rate in *P. hispanica* is highest in the NS and lowest in the septum, which contrasts with *T. mauritanica,* in which incorporation is highest at the MC. This contrasts further with the turtle, *T. s. elegans*, wherein incorporation is highest in the STR and the lowest in the MC (Font et al., 2001).

Another discrepancy is that lizards have considerably higher cell recruitment into the telencephalon compared to turtles, which thus far has gone unexplained.

The main and accessory OB undergo considerable neurogenesis relative to the rest of the brain in all reptiles studied thus far including snakes (personal observation; Font et al., 2001). There has been limited evidence that cells born in the sulcus ventrails/terminals tangentially migrate from the rostral forebrain to the OB in *T. mauritanica, Podarcis sicula, Psammodromus algirus* (the large psammodromus), *and* turtles (Font et al., 2001; Margotta et al., 1999; Peñafiel et al., 1996; Pérez-Cañellas and García-Verdugo, 1996). While this process is considered similar to the mammalian rostral migratory stream (RMS), the similarities still have only been investigated on a superficial level. Further study needs to be done to determine the mechanistic similarities between reptilian and mammalian RMS. For example, it has not been determined whether tubes of glial cells surround migrating neurons or whether there are guidance cues influencing or restricting migration in reptiles (Font et al., 2001).

Kaslin et al. (2008) expect that the extensive post-embryonic brain neurogenesis in non-mammalian vertebrates can be attributed to the continued development of pathways involving sensory systems after birth. They hypothesized that neurogenesis in fish, amphibians, and reptiles is due to the demand for increased CNS processing power in response to increased primary sensory input. Goss (1992) gave another explanation in which neurogenesis is not an adaptive function, but instead an ontogenetic residue of embryonic development that has

simply persisted in these taxa since the Paleozoic era. Self-repair of mild brain injuries or diseases could potentially be selected for, however it would require brain damage to be frequent enough that it could potentially influence fitness and selection (Goss, 1992). Gross (2000) hypothesized that adult neurogenesis is essential to mammalian learning and memory formation. This system may also be applicable to reptiles and warrants further exploration.

9. Plasticity and environment

Bird and mammal studies have demonstrated that several environmental variables affect adult neurogenesis, yet environmental studies in reptiles are severely lacking (Gould et al., 1998; Kempermann and Gage, 1999; van Praag et al., 1999). For example, seasonally breeding birds' testosterone levels are influenced by photoperiod. Exposure to longer days in springtime increases testosterone, which is associated with reductions in cell mortality and increases in brain volume in specific regions (Tramontin and Brenowitz, 2000).

Delgado-Gonzalez et al. (2011) investigated the role of season in proliferation and migration rates of new brain cells in *G. galloti*. They found that in autumn, winter, and especially spring, the quantity of BrdU labeled cells in the VZ were significantly greater after 30 days, implying they were not migrating to the parenchyma. However, in the summer, the ventricular zone lost considerable cells after the 30 day period due to migration. In *P. hispanica,* Ramirez et al. (1997) found that lizards maintained in long photoperiods had significant increases in proliferation. Short photoperiods, on the other hand, reduced the number of proliferating cells but did not affect migration.

Ramirez et al. (1997) also investigated effects of temperature in *P. hispanica.* They found cold temperatures led to a reduction in migration of the new cells, preventing cells from leaving the sulcus medialis and causing new cells to accumulate at the ependyma. Ramirez et al. also found 8h photoperiods reduced cell proliferation compared to 16h photoperiods. In *P. algirus* cold temperatures have been found to reduce proliferation 70-90% and reduce migration (Penafiel et al., 2001). Radmilovich et al. (2003) demonstrated significant increases in proliferation for warm-acclimated *Chrysemys d'orbigny* (black-bellied slider) turtles compared to cold-acclimated turtles. This contrasts with Marchioro et al. (2012) who found a reduction in migration rate but not proliferation due to cold temperatures in *T. hispidus.* In order to determine whether these phenomena can be generalized among all reptiles or how environmental variables like temperature and photoperiod impact neurogenesis, further studies investigating these variables should be conducted in snakes. Also, further studies must be done in the natural environment (Amrein et al., 2004) to confirm laboratory findings and provide the behavioral and environmental variables to better understand the "dynamics" of neurogenesis (Lindsey and Tropepe, 2006).

10. Regeneration in the adult telencephalon

Neuronal regeneration is a rare occurrence in the vertebrate CNS. In adult lizards, neuron proliferation and migration in the medial cortex are increased after lesioning with the neurotoxin 3-acetlypyridine (3AP) (Font et al., 1995b; Font et al., 1991; Kaslin et al., 2008). The damage from 3AP varies greatly among lizards of the

same species (for both *P. hispanica* and *Anolis carolinensis*) and between regions. For example, in *P.* hispanica 34% to 97% of neurons in the MC displayed pyknotic nuclei, but in other regions 3AP affected less than 50% of neuronal populations (Font et al., 1997b). Proliferation of microglia and radial glia increases in response to the damage so these cells can remove axonal and cellular debris (Font et al., 1995b; Lopez-Garcia et al., 1994; Nacher et al., 1999). Simultaneously, adult neurogenesis is upregulated and replacement cells begin to migrate from the VZ. After one to two weeks, neurons begin to enter and fill the void left by 3AP damage and completely replace the damaged area within a 6-8 week period (Font et al., 1991). Some replacement neurons establish synaptic contacts and join functional circuits (Molowny et al., 1995). By two months the previously damaged telencephalon is virtually indistinguishable from one that never received 3AP treatment (Font et al., 1997b). However, comparative studies using 3AP are limited because preliminary 3AP studies in snakes resulted in inconsistent distribution of damage (Font et al., 2001).

Minelli et al. (1977) found reactive proliferation in the cortical tissues of *Lacerta viridis* in response to surgical ablation, however the regeneration was not complete within 260 days. On the other hand, neuronal regeneration as a result of 3AP exposure is probably the most successful case of forebrain neurotoxic damage and neuronal regeneration in any vertebrate to date. While there are some examples of fish, amphibians, songbirds, and mammals that undergo induced regeneration or

reactive neurogenesis, lizards are the only tetrapods that can regenerate entire cerebral cortex regions bilaterally into adulthood (Font et al., 2001).

11. Future studies of adult neurogenesis in reptiles

Currently, the paucity of neurogenesis research in reptiles hinders the discovery of the evolutionary nature of neurogenesis (Lindsey and Tropepe, 2006). The reptilia are very diverse, and the paucity of snake studies has prevented the move from a collection of isolated studies to a comparative data set that can be used to identify meaningful patterns in adult neurogenesis. Furthermore, only one reptilian study investigates regional differences in neurogenesis over the organism's lifespan (Marchioro et al., 2005).

I propose that future studies need to be performed in snakes in the laboratory to determine baseline regional and developmental differences in adult neurogenesis. Then field studies should be conducted to determine changes in response to environmental variables including, but not limited to: (a.) changes in metabolic rate including those in response to digestion of prey and environmental temperature changes; (b.) scent and pheromone exposure; (c.) age; (d.) light cycle; (e.) exposure to stressors; and (f.) while gravid. The next chapter of this manuscript provides baseline laboratory data on neurogenesis in *P. regius*, to use as a comparative model species to study the evolutionary and physiological significance of adult neuroplasticity and neurogenesis. Additionally, the impact of neurogenesis on animal behavior must first be determined, so generalizations can be made and applied to

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understanding the correlations between human neurogenesis and behavior

(Boonstra et al., 2001).

Chapter 2. A BrdU study of proliferation, migration, and survival in the telencephalon of the ball python, *Python regius.*

Introduction

Adult neurogenesis is widespread throughout vertebrates and varies in presence and rate both among and within taxa (Barker et al., 2011). Over the past 20 years considerable research has focused on elucidating both the mechanisms and functions of adult neurogenesis. However, the overwhelming majority of neurogenesis studies have investigated the process in the mammalian brain, in which adult neurogenesis is very limited. Quantitative studies of neurogenesis in reptiles have focused on only a few lizard and turtle species. The lack of reptilian studies is both unexpected and detrimental to research not only because reptiles undergo extensive adult neurogenesis, but also because they are exceptional neural regenerators. Comparative studies of this trait among vertebrates may better clarify its evolution and function (Font et al. 2001; González-Granero, Lezameta, and García-Verdugo 2011). Relying on only a few models prevents the delineation of evolutionary trends (Lindsey and Tropepe, 2006). Further, comparison among several members of the same class can support patterns of structure and function within the given class (Rakic, 2002). For example, adult neurogenesis is absent in adult shrews (Bartkowska et al., 2008) and in the hippocampus of some bat species (Amrein et al., 2007), yet it is present in the six other mammalian orders investigated thus far (Barker et al., 2011).

Brain morphology and neural networks have diverged greatly among vertebrate classes as they have been shaped by natural selection. Several species have developed very specialized brain anatomy and physiology in response to environmental pressures (Butler and Hodos, 2005; Nieuwenhuyis et al., 1998; ten Donkelaar, 1988) while others have retained ancestral traits, thus providing extant evidence for tracing the evolution of morphology (Nomura et al., 2013b). Determining why some species have undergone selection for or against adult neurogenesis will further evolutionary research.

Reptilian brains exhibit a simple, laminated dorsal telencephalon with a 3-layered cortex, thought to be inherited from their amniote ancestry (Bruce, 2007; Wise et al., 2009). When Platel (1974) conducted morphometric studies of the developed reptilian brain he found continued growth of brain volume from young to old lizards. Examining *Podarcis hispanica* (Iberian wall lizards)*,* Lopez-Garcia et al. (1984) determined that the volumetric change throughout life was associated with continuous addition of newly generated neurons (Martínez-Guijarro and López-García, 1994). It is yet to be determined whether the increase in brain volume in reptiles is also associated with increases in neuron size, quantity, or spacing. The primary function of adult neurogenesis in reptiles may be supplying the brain structures with new cells as the structures enlarge during development (Barker et al., 2011).

Snakes diverged from lizards approximately 92 million to 161 million years ago (Gorr et al., 1998). Among snakes, pythons are the most basal extant

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macrostomatan species (Castoe et al. 2011; Slowinski and Lawson 2004). The basal

origins of these snakes may provide insight into the evolutionary origins of adult

neurogenesis and adult brain development. Pythons exhibit many unique traits that

can further our understanding of adult neurogenesis from a comparative perspective.

Most are constricting sit-and-wait predators that consume large prey relative to their

body size, resulting in metabolic changes and plasticity of other physiological

systems, including the digestive and cardiovascular systems (Andersen et al., 2005;

Secor and Diamond, 1998). Some species of female pythons also maintain the

temperature of their eggs by shivering thermogenesis when brooding. Shivering

thermogenesis occurs when a series of muscular contractions of the whole body

generates heat for incubating eggs (Harlow and Grigg, 1984; Stahlschmidt and

DeNardo, 2008; 2009; 2010; Vinegar et al., 1970). Pythons experience vomeronasal

chemical sensing and possess labial pits for infrared detection (Molenaar, 1978a;

Molenaar, 1978b).

Python regius (commonly known as "Ball Pythons" or "Royal Pythons") are

native to western Africa, but are commonly bred as exotic pets internationally. They

are very docile and easy to handle without risk of injury to the handler or the snake.

They do not require intricate laboratory housing and can be maintained by giving

them access to water *ad libitum*, a heating device, and regular access to feeder

mice. In fact, contrary what many researchers might expect, pythons are easier to

handle and maintain than rats (Secor et al., 2001).

In two experiments the following were determined: (a) the dose of the cell birth marker, 5-bromo-2'-deoxyuridine (BrdU), that best marks proliferating cells in the *P. regius* telencephalon, (b) the brain regions in which telencephalic proliferation occurs, and (c) the rate of migration and survival of the newborn cells. In accordance with previous studies (Cameron & McKay, 2001; Hancock et al., 2009), higher doses of BrdU should mark more newborn cells than lower doses. Based on previous studies of squamates, I expected higher cell proliferation and migration at the anterior olfactory bulb (AOB), medial cortex (MC), dorsoventricular ridge (DVR), and nucleus sphericus (NS) than in other regions (Font et al., 2001; Pérez-Cañellas and García-Verdugo, 1996).

Results of this study will provide groundwork for future studies investigating the evolutionary trends pertaining to environmental, hormonal, and age-related effects on adult neurogenesis. Future studies may provide evidence for whether properties unique to snake physiology affect neurogenesis, and determine the relationships among telencephalic proliferation, migration, and survival.

Materials and Methods

Experiment 1:

Procurement and treatment

The use and treatment of snakes was conducted with the approval of the California Polytechnic State University IACUC protocol No. 915. Fifteen male *P. regius* ($\bar{x}$ = 95.92g ± 43.03g) were purchased from Reptile Industries (Naples, FL)

and delivered on July 8, 2011. Snakes were acclimated in the laboratory for for 14

days. During this period they were housed in 26 x 45 x 36 cm clear plastic containers

(Monoflo) on a 12:12 light:dark cycle. Each container had a terra cotta water dish, an

ABS pipe cut in half lengthwise to serve as a "shelter" and newspaper lining the

bottom for absorption and cleaning.

Each container was marked with the corresponding number and placed

randomly on a cage rack. In order to maintain similar metabolic rates among all

snakes during treatment, snakes were fed mice selected according to proportional

weight on day 3 ($\bar{x}$ = 2.77%m/m ± 0.21%) except for snake #3, which refused to eat.

Snakes were haphazardly placed into stratified groups to avoid a possible effect of

mass on treatment (Table 1). On day 15 they were subcutaneously injected with

BrdU dissolved in 0.9% NaCl at 20mg/mL. Volumes were injected according to the

following 3 treatments: 50mg/kg, 100mg/kg, or 250mg/kg Bromodeoxyuridine (BrdU;

Sigma Aldrich).

Table 1: Stratified random placement of snakes by mass (g) into one of three
treatment groups with different dosages of bromodeoxyuridine (50, 100, or 250 mg
BrdU/kg body weight).

50mg/kg		100mg/kg		250mg/kg	
Snake	Mass (g)	Snake	Mass (g)	Snake	Mass (g)
3	744.73	8	653.23	4	624.82
2	888.78	1	721.41	5	656.74
14	1006.16	13	996.19	11	878.97
12	1038.05	10	1067.25	9	1003.04
7	1089.52	6	1149.65	15	1232.29
Mean	**953.45**		**986.71**		**879.17**
SE	*61.72*		*86.84*		*112.74*

Sacrifice and perfusion

Twenty-four hours after BrdU treatment, snakes were deeply anesthetized via isofluorane inhalation and were transcardially perfused with 0.9% NaCl, 0.1% NaNO$_2$, 0.1M phosphate buffer (PB) wash followed by 4% paraformaldehyde in 0.1 M PB, pH 7.2, with 0.1% NaNO$_2$. Following perfusion, skulls containing brains were extracted and placed in 4% paraformaldehyde for at least 2 hours. Brains were then carefully removed from the skulls to postfix for 24 h in 4% paraformaldehyde followed by 24 h in 0.1 M PB solution. An example of a removed, fixed, complete brain is shown in figure 3. Brains were weighed and placed in gelatin overnight until the gelatin formed semisolid blocks around the tissue. Then the blocks were placed in 4% paraformaldehyde for 24 h and then 30% sucrose until they sank, frozen in dry ice, and stored at -80˚C until sectioning.
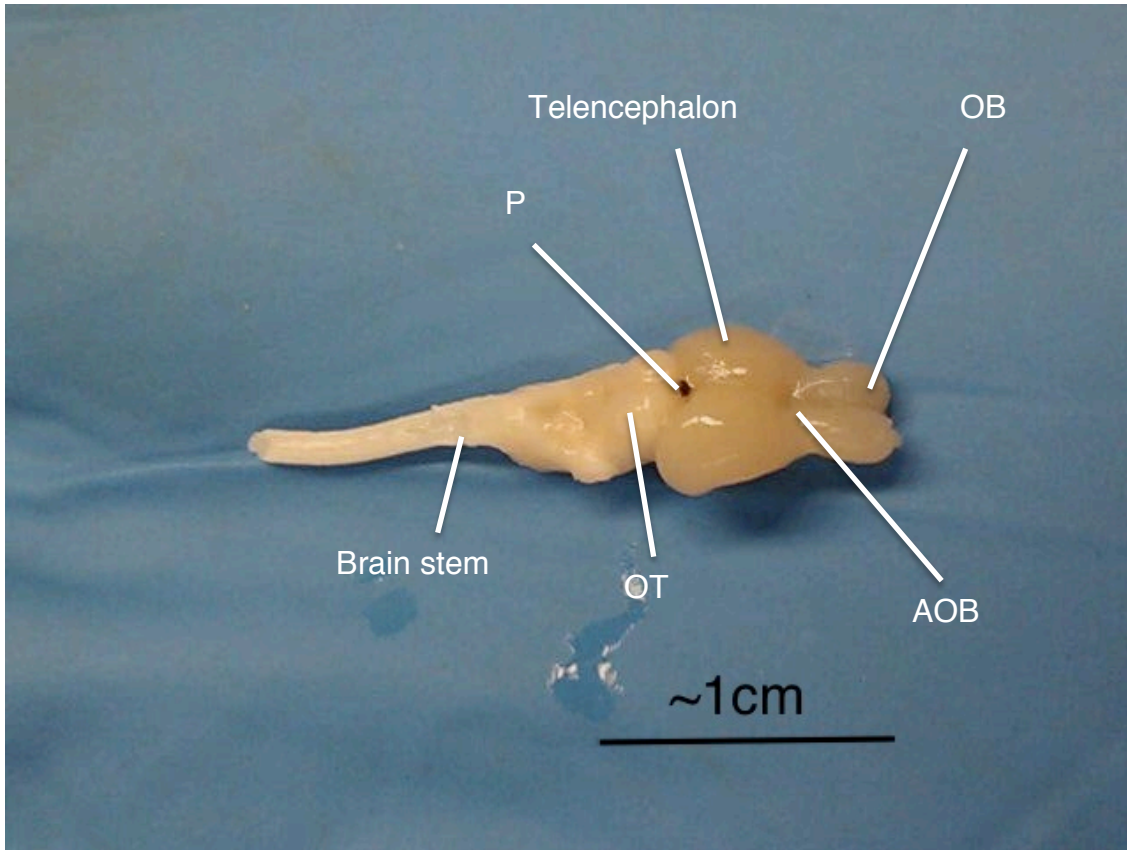
Figure 3: An extracted, perfused, complete *P. regius* brain. The dorsal side of the telencephalon (pallium) is shown. AOB: accessory olfactory bulb, OB: olfactory bulb, OT: optic tectum, P: pineal gland.

Sectioning and histochemistry

Four series of 40μm thick parallel coronal sections were obtained using a Bright OTF-5000 cryostat. One series (every fourth section) was directly mounted to slides, hydrated with mounting solution, allowed to dry, and stained with cresyl violet. Cresyl violet stained sections were photographed with a Leica EZ4 stereo microscope at 10x magnification and stored as digital images for measurement and identification of brain regions of interest (fig. 4).
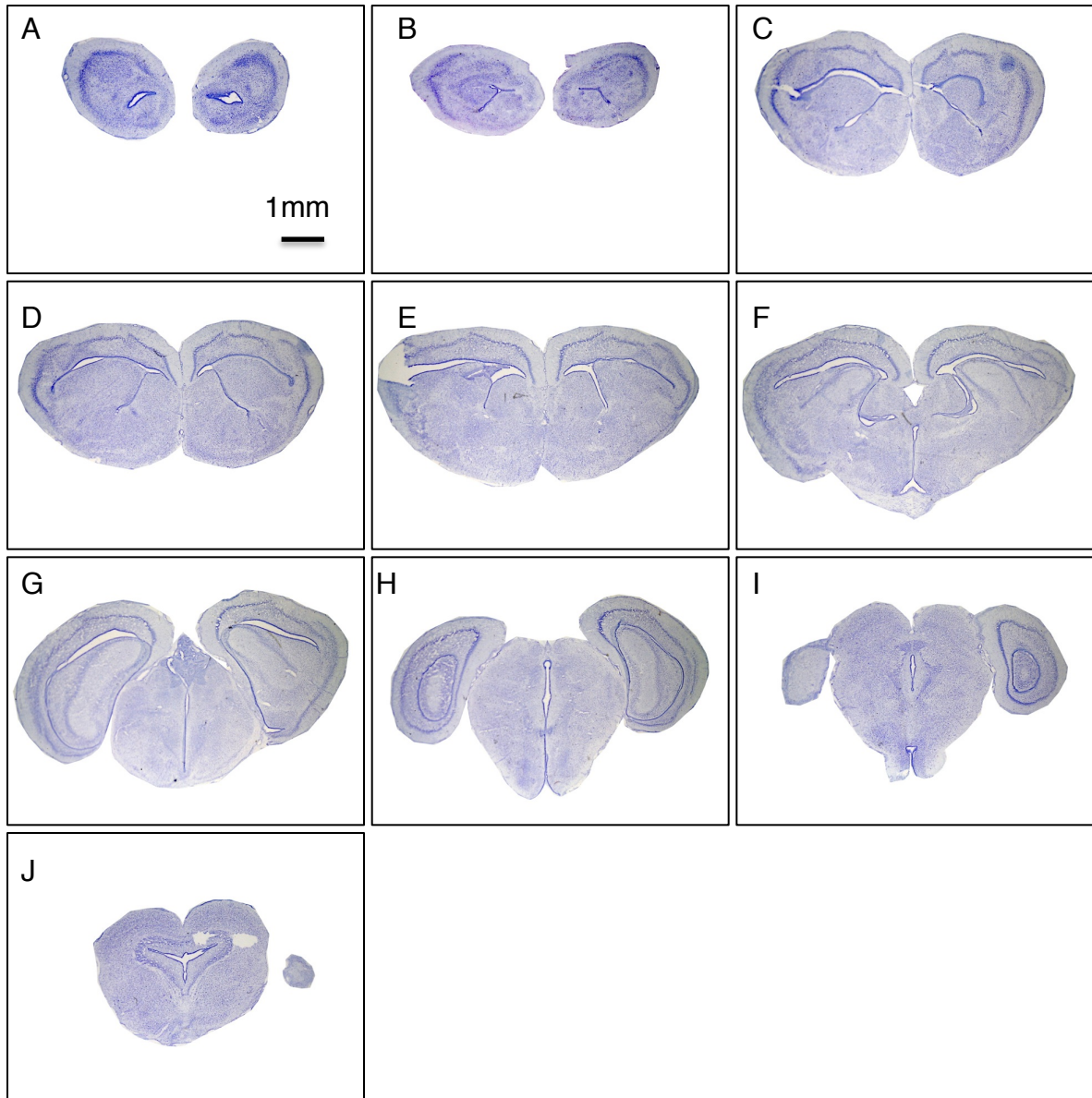
Figure 4: The stack of images taken from cresyl violet stained sections (every 16th section shown) of snake #7 used to create a 3D model and determine study regions for experiment 1.

The other sections were stored in cryoprotectant at -20˚ until they were ready

to be used. Free-floating sections were immunostained for BrdU as follows: Every

eighth section was washed with phosphate-buffered saline (PBS) 3 times for 5

minutes prior to DNA denaturation in 4N HCl for 15 minutes. After denaturation,

sections were rinsed for 5 min in PBS and neutralized in 3.8% sodium borate wash

(pH 8.5) for 10 min. Sections were rinsed 3 times for 10 minutes in PBS and

immediately blocked in a solution of 5% normal horse serum (Vector Laboratories

Inc), 1% bovine serum albumin, and 0.5% H_2O_2 in PBS + 0.3% Triton X-100 (PBST).

Immediately following blocking, sections were placed in a 1:1,000 anti-BrdU (clone:

Bu20a, isotype: IgG1, kappa, code: M0744, DakoCytomation, Glostrup, Denmark)

dilution in PBST and left on a rotator table for 24 h at room temperature. Another

series of three 5-min washes in PBST was followed by 1 h of incubation in

biotinylated anti-mouse secondary antibody (1:100; Vector Laboratories), three 5-

minute washes in PBST, and a 1h incubation in avidin-biotin-peroxidase complex

(PK-6100, Vector Laboratories). Finally, sections were washed 3 times for 5 minutes

in PBST followed by detection of bound peroxidase complexes via 4-minute

incubation in Vector SG Chromagen (Cat. No.SK-4700, Vector Laboratories). The

reaction was stopped by two 5 min washes in PBS. Sections were then mounted

onto slides and coverslipped.

Measurement

To define study regions, a digital 3-dimensional model was created from the

captured images according to the following process: Images were masked using

Photoshop CS6 13.0 and transformed to align with adjacent section images in a

stack (supplemental movie 1: http://youtu.be/Lw8nD2V0FrI). The stack was migrated

to ImageJ (NIH, USA) and converted to a 3D model using Java3D. The 3D model

and 2D stack were used to determine locations where rostral-caudal morphological

changes occur and clearly define these regions so counting would be consistent between brain sections (fig. 5).

AOB

1

2

3

4

5
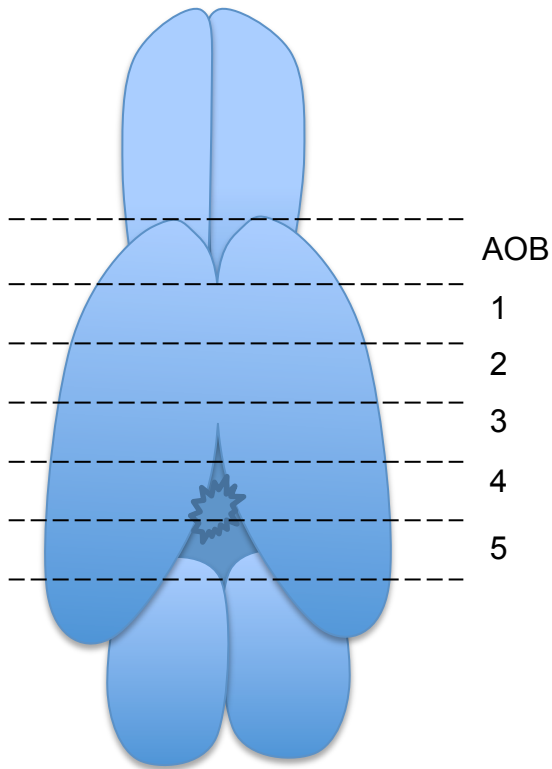
Figure 5: Predetermined regions defined for *P. regius* telencephalon. Regions were divided according to rostro-caudal landmarks (1-5). AOB: Accessory olfactory bulb. Rostral-caudal regions defined according to morphological changes to determine areas where changes in cellular density occur. Then regions with similar and consistent cell densities were used for the study area.
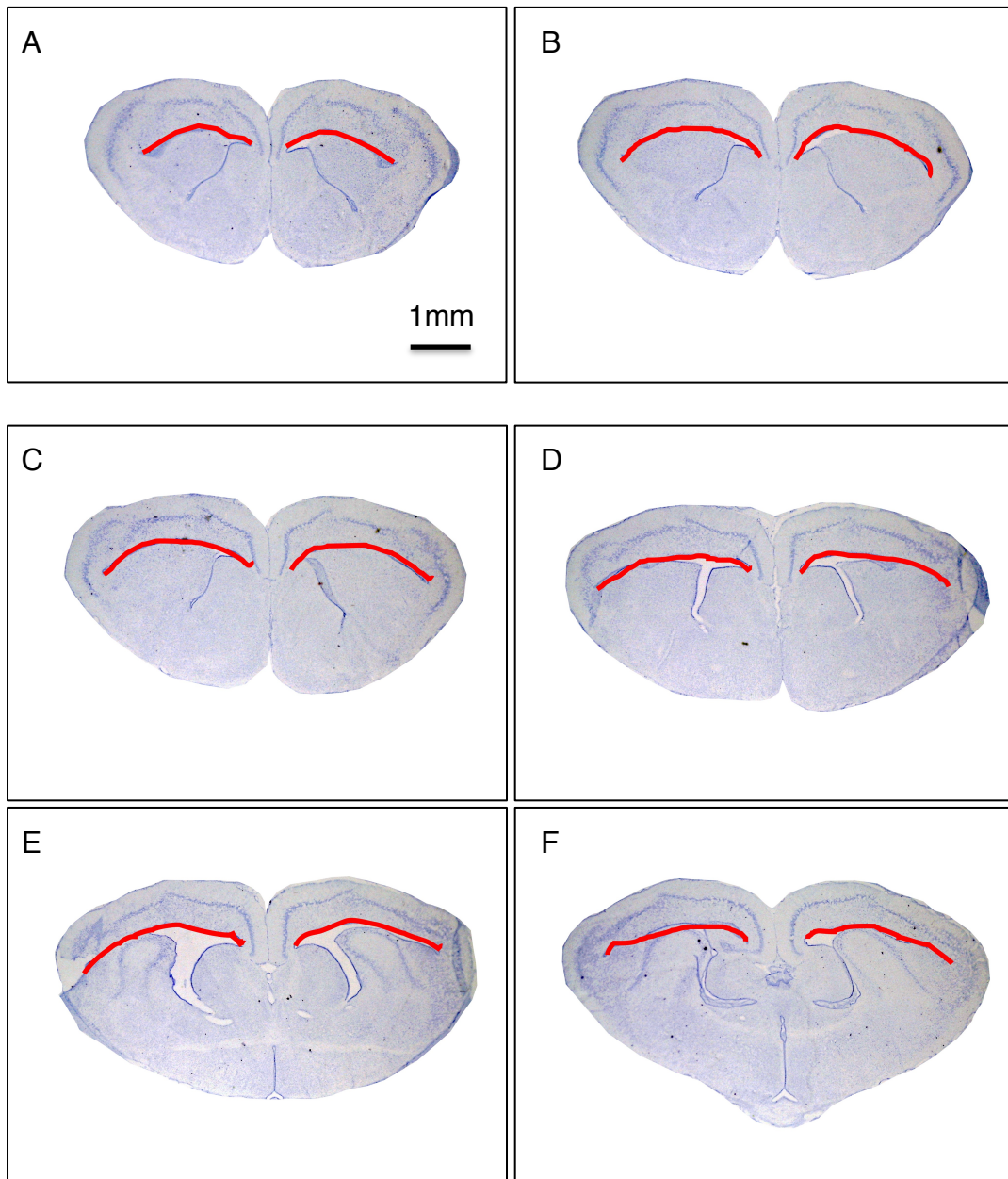
Figure 6: Supraventricular ependymal surface area used for counting cell density for 3 BrdU treatments.

BrdU-immunoreactive (BrdU-ir) cells were observed under an Olympus CH series light microscope at 400X and counted along the left and right ventricle along

the ependymal lining from the sulcus septomedialis to the sulcus lateralis for each within each section (fig. 6).

To determine how cell counts and line measurements would be performed to calculate cell density for statistical analysis, cells along the measured line were first counted in the 250mg/kg group. Regions 1-3 were chosen for statistical analysis because they had large enough cell counts to clearly reveal any potential differences among the three treatments (fig. 7; supplemental movie 2: http://youtu.be/Ka7VirggVPM). Regions 4-5 were excluded because their cell counts were too low to show differences, and because any differences would likely be due to differences in ependymal length measurement instead of cell quantity. The AoB was excluded because the cell counts were too inconsistent within the region and the counts were exceptionally high compared to the rest of the telencephalon.

New cell density was determined for each region by measuring the length of the ependymal lining from the sulcus septomedialis to the sulcus lateralis and by counting anti-BrdU marked nuclei along the measured line (fig. 6). The ependymal layer was defined as the cell layer lining the ventricles and two-to-three cell body diameters from the ventricles. The cell counter was blind to treatments.

Cell densities throughout each region were calculated as follows:

$$regional\ cell\ density = \left(\frac{\sum marked\ cells\ from\ all\ sections\ in\ region}{\sum line\ length\ from\ all\ sections\ in\ region}\right)$$

For determination of overall cell density across all three rostral-caudal regions, 1-3, the following calculation was used:

$$overall\ cell\ density = \left(\frac{\sum marked\ cells\ from\ all\ sections\ in\ all\ 3\ regions}{\sum line\ length\ from\ all\ sections\ in\ all\ 3\ regions}\right)$$
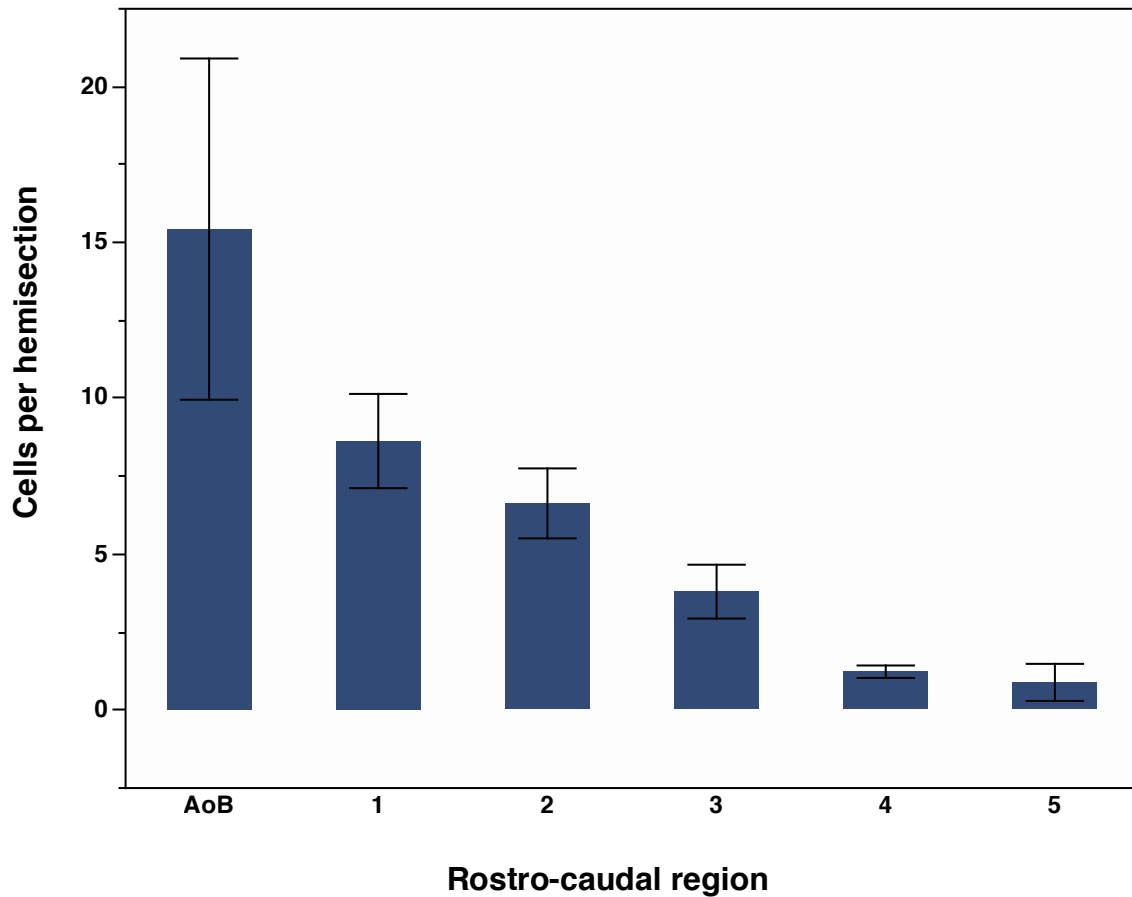
Figure 7: Count of new cells per hemisection (mean +/- SE) within each rostro-caudal region, for the 250mg/kg treatment group.

Statistical analysis

An ANOVA was performed using JMP 10.0 (SAS, 2013) to determine the

effect of dosage on cell marking along the selected ependymal surface. Data

followed a normal distribution with equal variances and was left untransformed.

ANOVA was followed by a post-hoc Tukey-Kramer HSD. All differences were considered significant at α < 0.05.

Experiment 2:

Procurement and treatment

Procurement and treatment were performed similarly to experiment 1 and in compliance with California Polytechnic State University IACUC protocol No. 915. Fifteen juvenile male *P. regius* ($\bar{x}$ = 121.44 g ± 5.83 g) were delivered on February 2, 2012. During the 17-day acclimatization period snakes were fed mice on day 2 ($\bar{x}$ = 11.28% m/m ± 0.21% m/m) and day 14. After the acclimatization period, snakes were weighed and haphazardly placed in three treatment groups (2 month, 2 week, 2 day) with to prevent a possible effect of mass on treatment (table 2). Snakes in the "2 month" group received 100mg/kg BrdU 2 months before sacrifice, snakes in the "2 week" group received 100mg/kg BrdU 2 weeks before sacrifice, and snakes in the "2 day" group received 100mg/kg BrdU 2 days before sacrifice (fig. 8). During the 2 month treatment period were fed mice on day 13 ($\bar{x}$ = 10.03% m/m ± 0.19% m/m), day 27 ($\bar{x}$ = 11.32% m/m ± 0.32% m/m), and day 40 ($\bar{x}$ = 9.85 % m/m ± 0.17 % m/m).
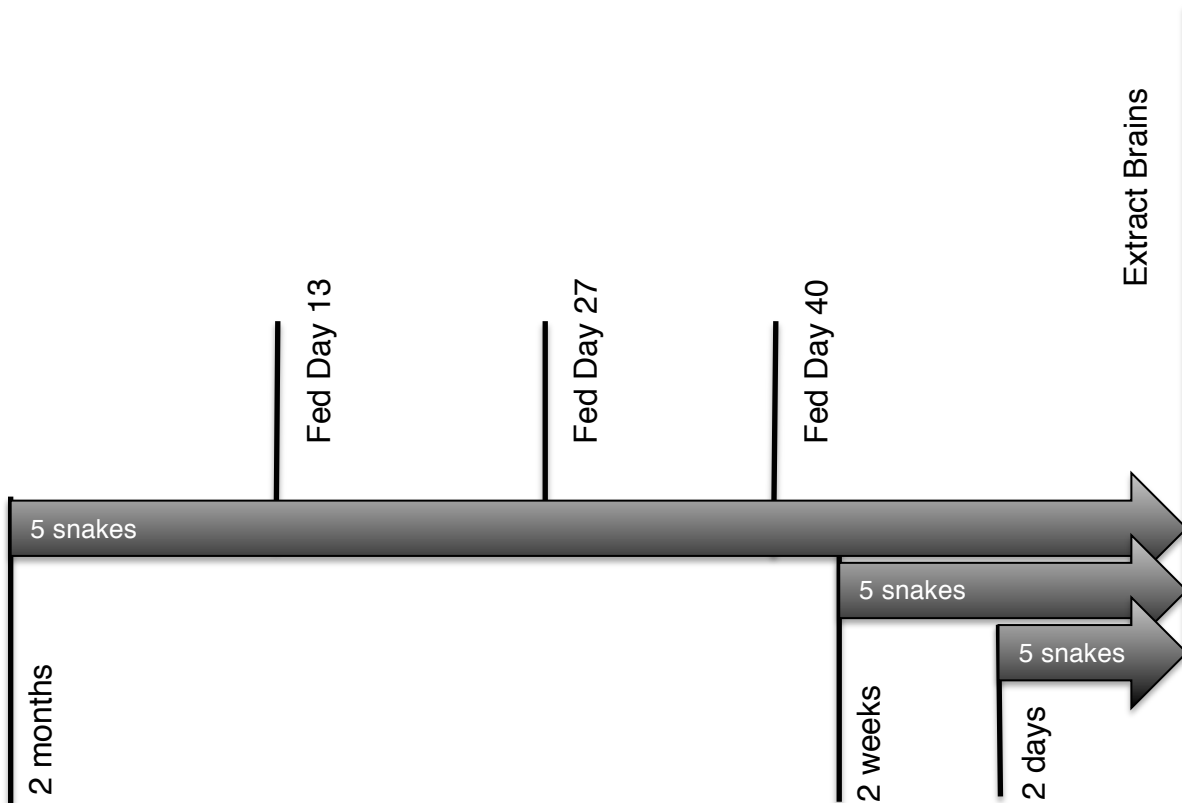
Figure 8: Timeline of Experiment 2. Snakes were sacrificed 2 months, 2 weeks, and 2 days after BrdU injection.

Table 2: Stratified placement of snakes into treatment groups at beginning of study.

2 day		2 week		2 month	
Snake	Mass (g)	Snake	Mass (g)	Snake	Mass (g)
11	102.4	4	96.6	8	103.3
10	117.6	15	98.3	1	108.6
13	131.5	5	98.5	2	128.5
7	140.8	14	142.7	3	131.8
6	106.6	2	176.3	9	138.1
Mean	**119.78**		**122.48**		**122.06**
SE	*7.28*		*16.02*		*6.81*

Sacrifice and perfusion

Upon completion of the 60-day treatment period, snakes were sacrificed, perfused, and whole brains were stored following the protocol described in experiment 1.

Sectioning and histochemistry

Four series of $36\mu m$ thick parallel coronal sections were obtained by cryostat. A cresyl violet series was mounted according to the methods stated in experiment 1. Cresyl violet stained sections were photographed at 10x with a Leica EZ4-D, and photos were digitally stored as "jpeg" images. Anti-BrdU fluorescence immunohistochemistry was performed on free-floating sections as follows: Every eighth section was washed in PBST three times for 10 min and placed in 4N HCl for 15 min. Sections were rinsed in PBS for 5 min and neutralized for 10 min in 3.8% sodium borate wash (pH 8.5). Then they were placed in 5x "animal free blocker" diluted at 1:5 in PBS (SP-5030, Vector Laboratories) for 1 hour. Blocking was followed by immediate placement of sections in a 1:1,000 dilution of mouse anti-BrdU (clone: Bu20a, isotype: IgG1, kappa, code: M0744, DakoCytomation, Glostrup, Denmark) in PBST for 24 hours shaking on a rotator table. Thereafter sections were washed three times in PBST for 10 min and placed in 1:200 FITC horse anti-mouse IgG antibody (FI-2000, Vector Laboratories) covered on a rotator table for 1 hour. Lastly sections were placed in 3, 10 minute PBST washes, mounted on slides, and coverslipped with Vectashield hard set mounting medium with DAPI (H-1500, Vector Laboratories).

Measurement

To determine study regions, sections were compared to those in Halpern (1980) and Smeets (1988), and regions were determined by direct observation of cresyl violet stained cells under the microscope at 400x (fig. 9). Masked images were stacked in imageJ and Java 3D modeling was used to create a 3-dimensional model (fig. 11 and supplemental movie 3: http://youtu.be/l995WMN8mK8). The 3D model was then used to determine and demarcate the study regions including the accessory olfactory bulb (AOB); anterior dorsal (AD) and anterior ventral (AV) regions (retrobulbar regions) including the anterior-most TUB and ACC in the AV region; ventral lateral (VL) and ventral medial (VM) regions containing the central and posterior TUB, ACC, and the anterior commissure in the VM region, and the STR and accessory olfactory tract in the VL; dorsoventricular ridge (DVR); septum (S); anterior dorsal, lateral, and medial cortex (aDC, aLC, and aMC); posterior dorsal, lateral, and medial cortex (pDC, pLC,, pMC); anterior and posterior nucleus sphericus (aNS, pNS); and posterior cortex (PC).
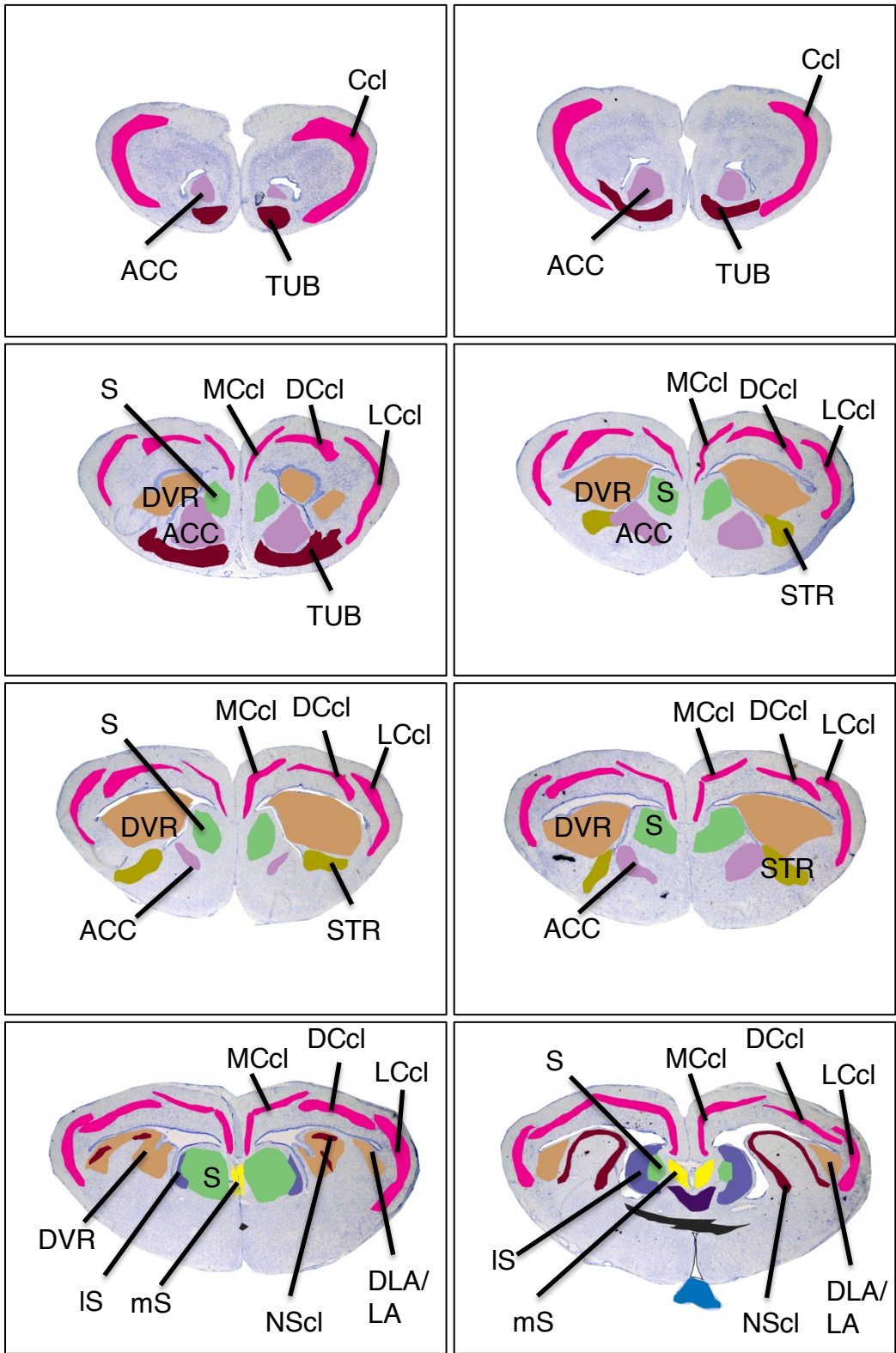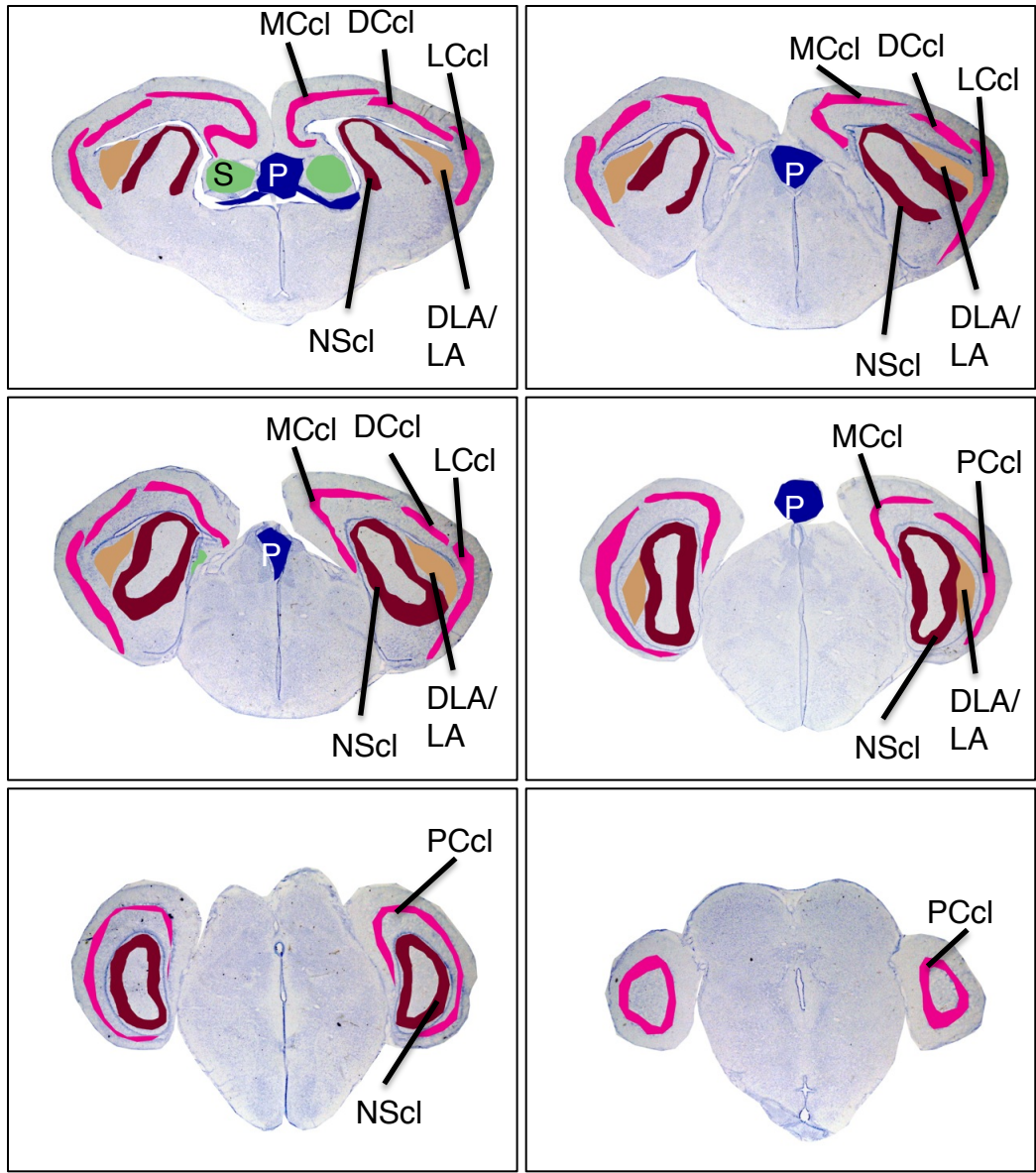
Figure 9: Every 16th section taken from python number 11, marked with colors depicting cellular nuclei regions determined by observing cellular changes in cresyl violet stained tissues. ACC = nucleus accumbens, TUB = olfactory tubercle, Ccl = cortical cell layer of the retrobulbar region, MCcl = medial cortex cell layer, DCcl = dorsal cortex cell layer, LCcl = lateral cortex cell layer, PCcl = posterior cortex cell layer, STR = striatum, DVR = dorsal ventricular ridge, S = septum, IS = lateral septal nucleus, mS = medial septal nucleus, P = pineal gland, NScl = nucleus sphericus cell layer.

The cresyl violet series was used to determine regional area (RA) for all

sections. The area of each region on each section was measured with the freehand

tool in ImageJ. Anti-BrdU stained sections were observed under a fluorescence microscope and compared to corresponding cresyl violet sections. Cell counts were performed in the predetermined regions (fig. 11). Cells were categorized as ependymal if they were within 3 cells of a ventricle, or parenchymal if they were at least 3 cells away from a ventricle. Regional cell density was determined for each snake according to the following calculation:

$regional\ ependymal\ cell\ density$

$$= \frac{\sum ependymal\ cells\ across\ all\ sections\ in\ a\ given\ region}{\sum RA\ from\ all\ sections}$$

or

$regional\ parenchymal\ cell\ density$

$$= \frac{\sum parenchymal\ cells\ across\ all\ sections\ in\ a\ given\ region}{\sum RA\ from\ all\ secctions}$$
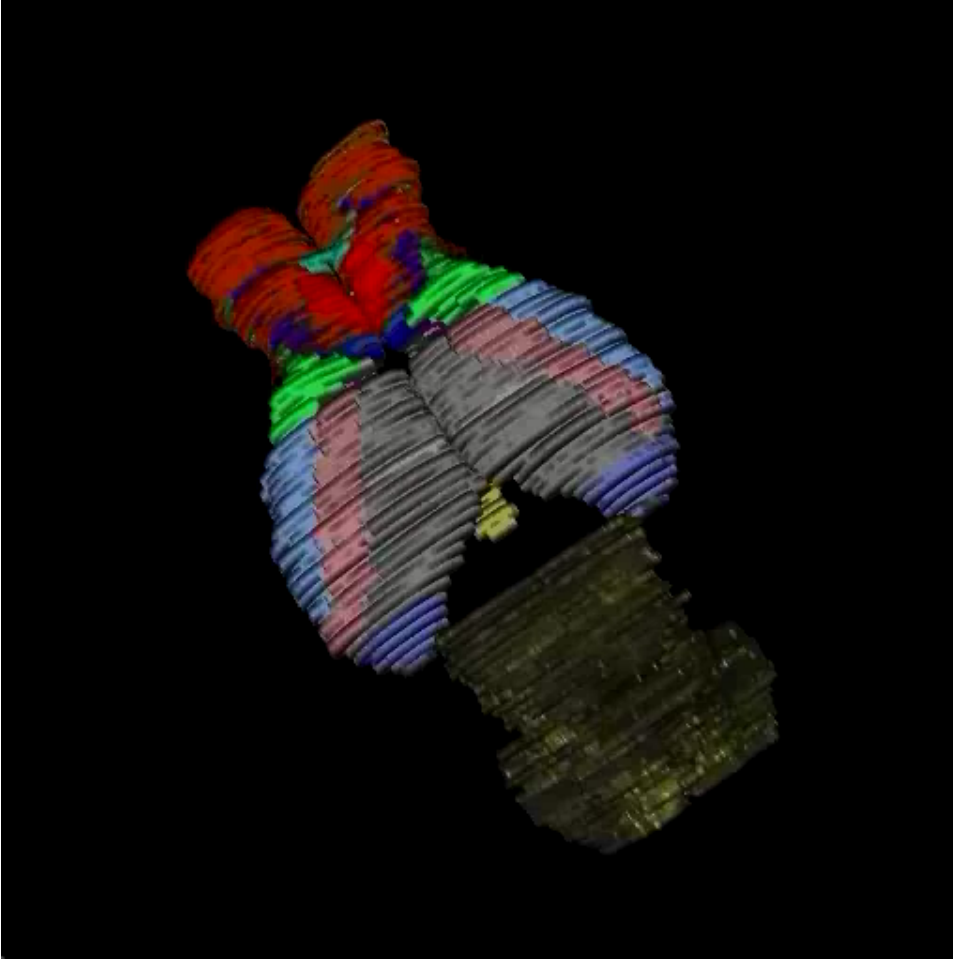
Figure 10: *P. regius* brain reassembled as a 3D image in ImageJ using Java3D for experiment 2. Colors only denote different regions and no other factors.

Regional ependymal cell density was used as an indicator to determine 2-day proliferation rate. In order to make comparisons between regions and treatments, mean values and standard errors were calculated from regional density values in the same treatment groups.

D

LC
DC
MC
DVR
S
VL
VM

E

LC
DC
MC
DLA/
LA
NS
S
VL
VM

F

MC

DC

DLA/
LA

LC

NS

S

P

D

G

MC

DC

LC DLA/
LA

NS
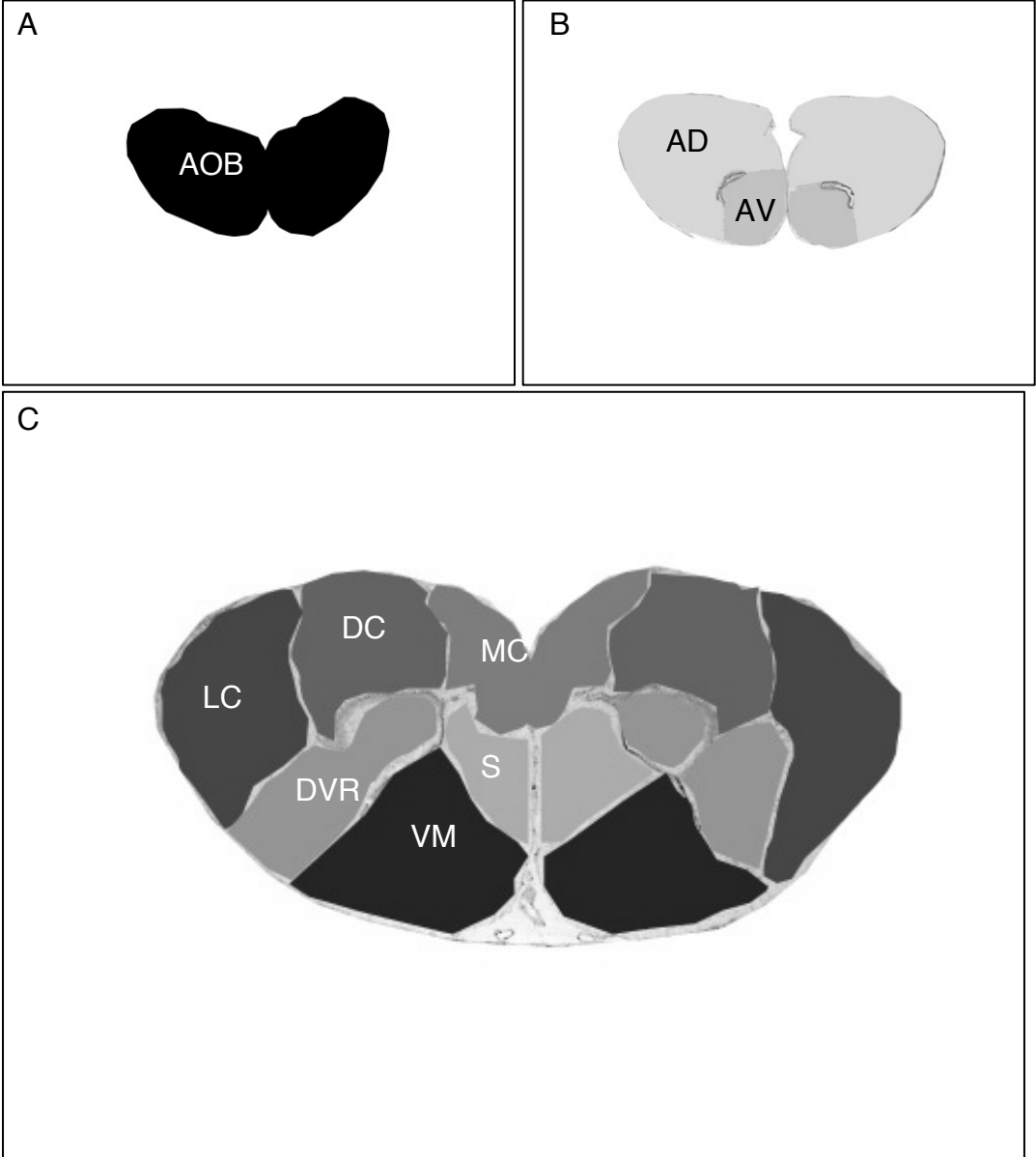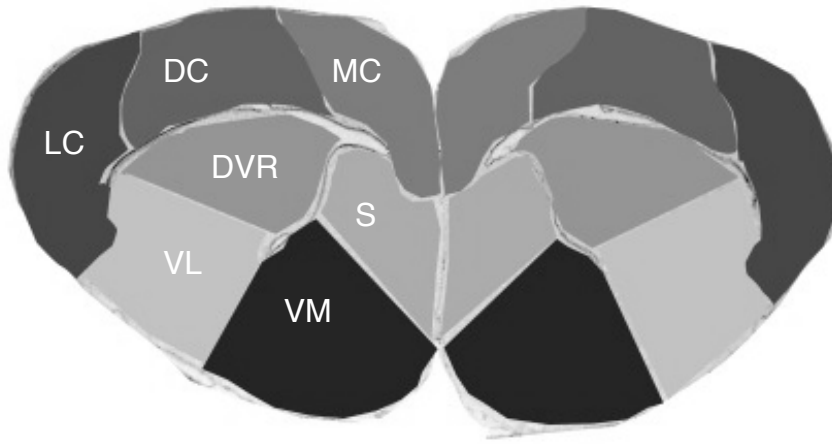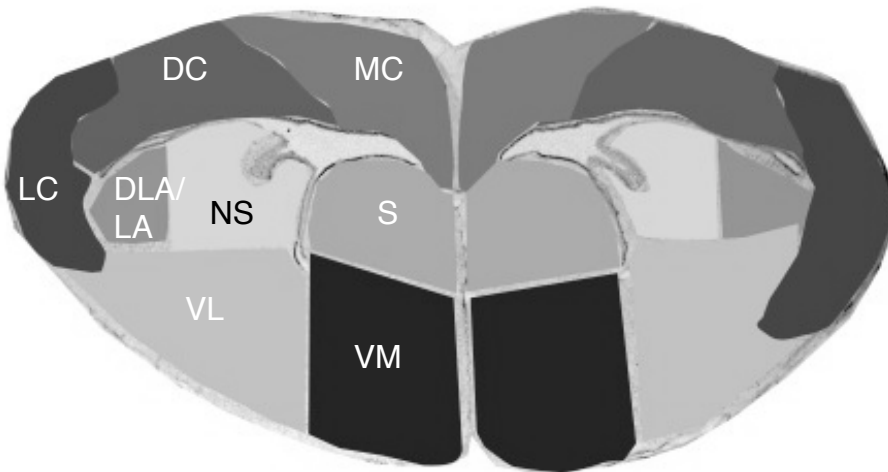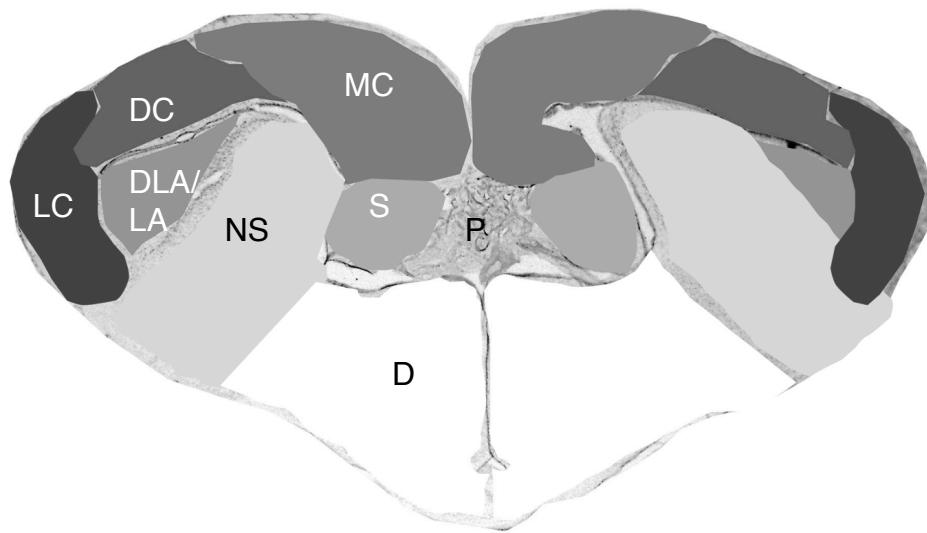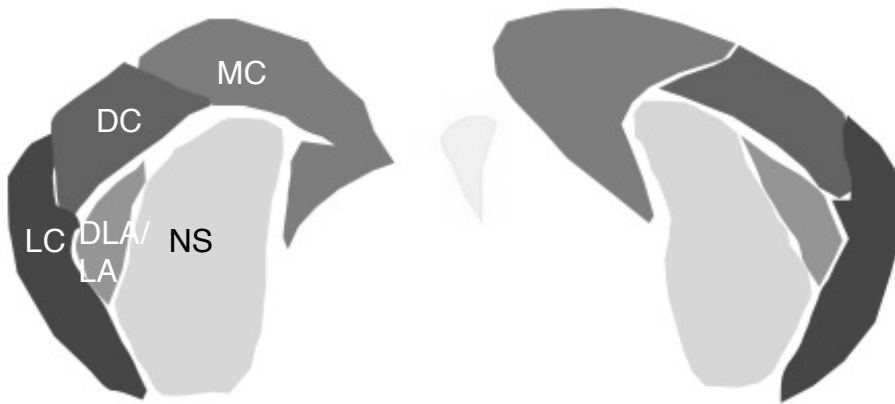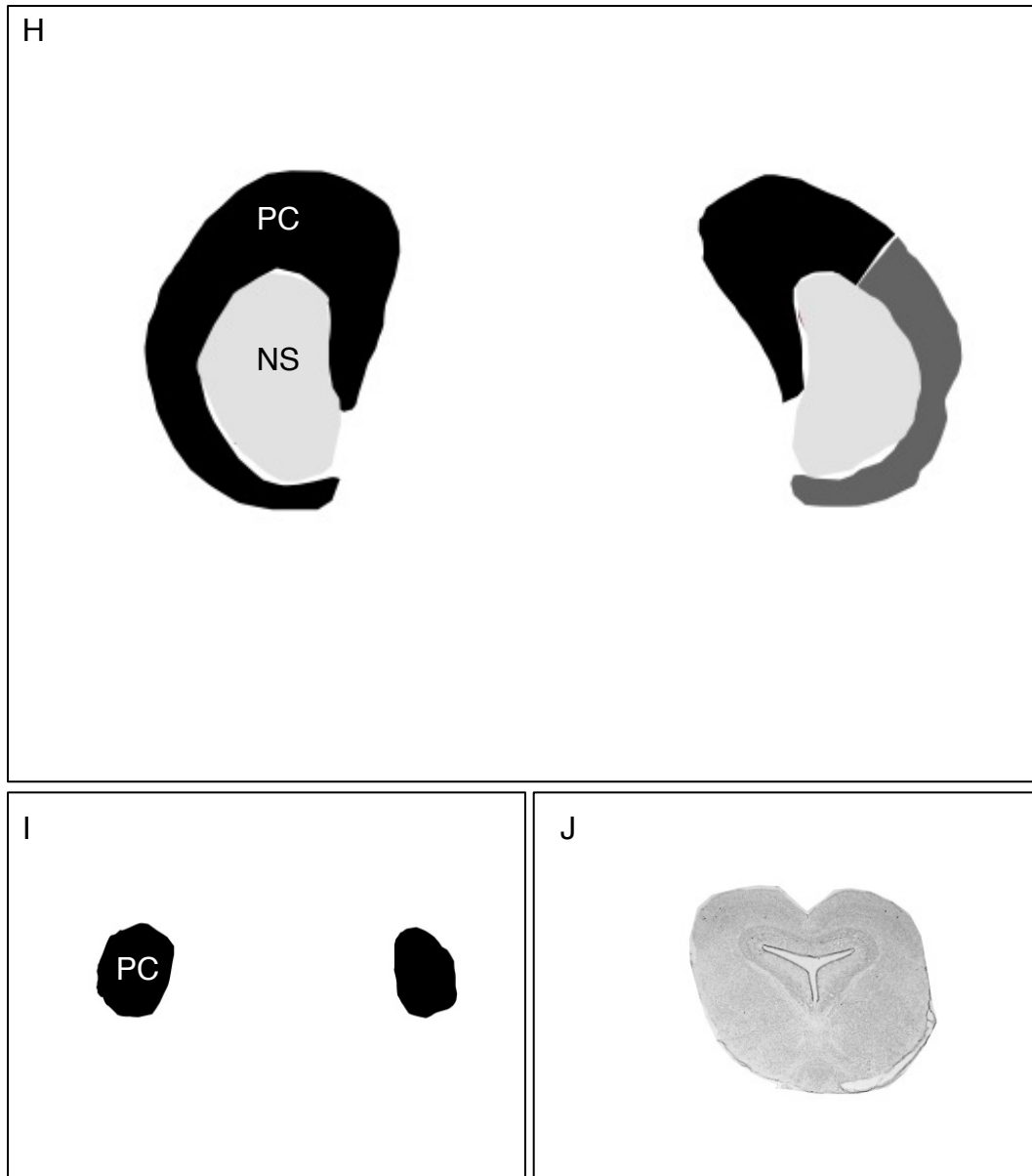
Figure 11: Every 16th section taken from a representative python cresyl violet stained, photographed and labeled using Photoshop CS3. Study regions determined for cell counts are highlighted. Regions correspond with quantification data in figs. 11, 12, and 13.

To determine the percentage of the originally proliferating cells that migrate into the parenchyma and survive, mean cell densities were calculated from complete regional snake brain data for all three treatments. Then the following calculations were performed:

$$\%migration_{2\ weeks} = \frac{mean\ regional\ parenchymal\ cell\ density_{2\ weeks}}{mean\ regional\ ependymal\ cell\ density_{2\ days}}$$

$$\%survival_{2\ months} = \frac{mean\ regional\ parenchymal\ cell\ density_{2\ months}}{mean\ regional\ ependymal\ cell\ density_{2\ days}}$$

In several regions, cells were marked in the parenchyma after only 2 days. These cells are likely of endothelial origin and they were not counted for the 2-day treatment. Since ventricular endothelial cell turnover has been determined for adult rats and mice as approximately 20 hours (Schultze & Korr, 1981; Korr et al., 1975, respectively), it is likely that any endothelial cells potentially marked with BrdU in the parenchyma in the 2 week and 2 month treatments would no longer exist at the time of sacrifice. Thus, only ependymal, not parenchymal cells were counted at 2 days. But all parenchymal cells were subsequently counted throughout for the 2 week and 2 month groups.

Results

Experiment 1

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Treatment had a significant effect on ependymal nuclei marking (overall cell density) ($F = 5.69$, d.f. = 2, 9, $p = 0.025$; Fig. 12). A post-hoc Tukey-Kramer HSD confirmed a significant difference between the 50mg/kg treatment and 250mg/kg treatment ($p = 0.026$; fig. 12). There was no difference between the 50mg/kg treatment and the 100mg/kg treatment ($p = 0.784$) or the 100mg/kg treatment and the 250mg/kg treatment ($p = 0.116$).
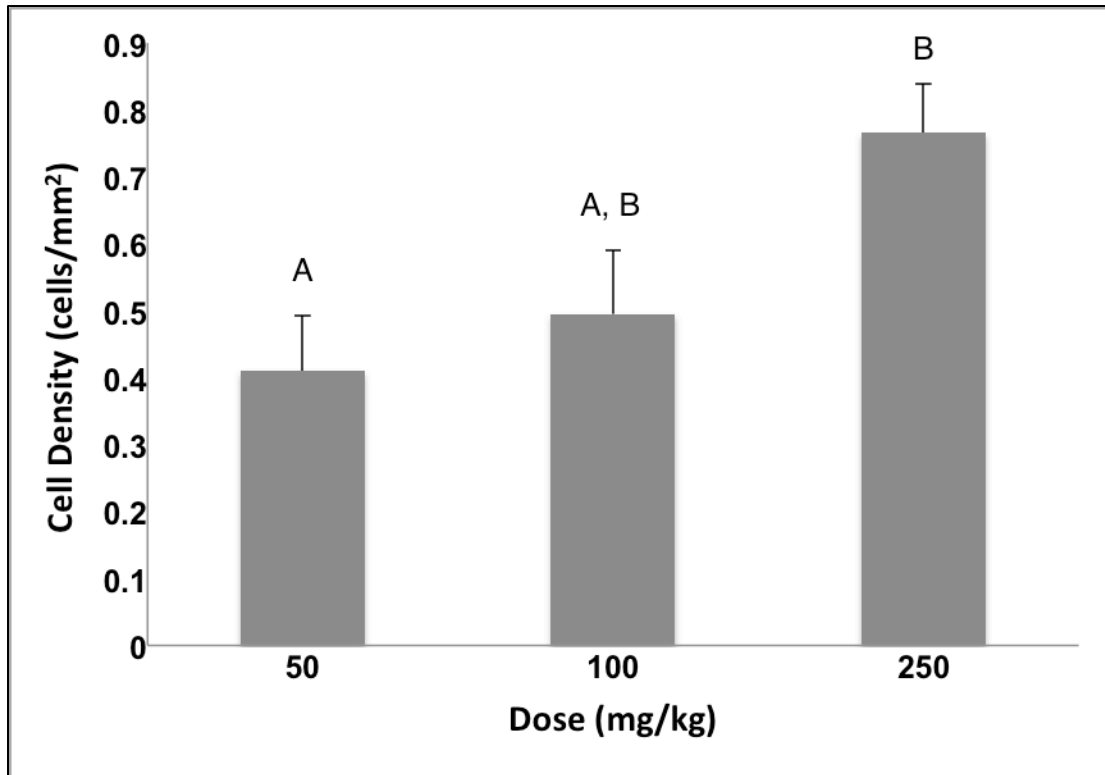
Figure 12. Mean cell new cell density (cells/mm^2; ±SE) in the supraventricular ependymal zone for snakes exposed to 50mg/kg, 100mg/kg, or 250mg/kg BrdU.

Experiment 2

Cell proliferation was highest in the retrobulbar regions (AD, AV), the AOB, DLA, DVR, pNS, S, and pNS (fig. 13A, B). After 2 weeks, cells migrated most to the parenchyma of the retrobulbar regions, the AOB, VL, DVR, and aNS (fig. 13A, C). After 2 months, the new cells survived most in the parenchyma of the AOB, AV, DLA, DVR, and AD (fig. 13A, D). Regions with the highest migration (%migration$_{2weeks}$) were the aNS, aMC, aLC, VL, DVR, VM, AD, and AOB (fig. 14). Regions with the highest survival (%survival$_{2\ months}$) were the aLC, AOB, aNS, aMC, DVR, aDC, VM, and pLC (fig. 15). Even after 2 months, new ependymal cells did not migrate and remained in the ventricular zone of several regions (fig. 16). The regions

with the greatest ependymal-cell-to-parenchymal-cell ratio after 2 months were the

pDC, DLA/LA, aMC, and pLC.

B

2 Day

C

New Cell Density (cells/mm3)
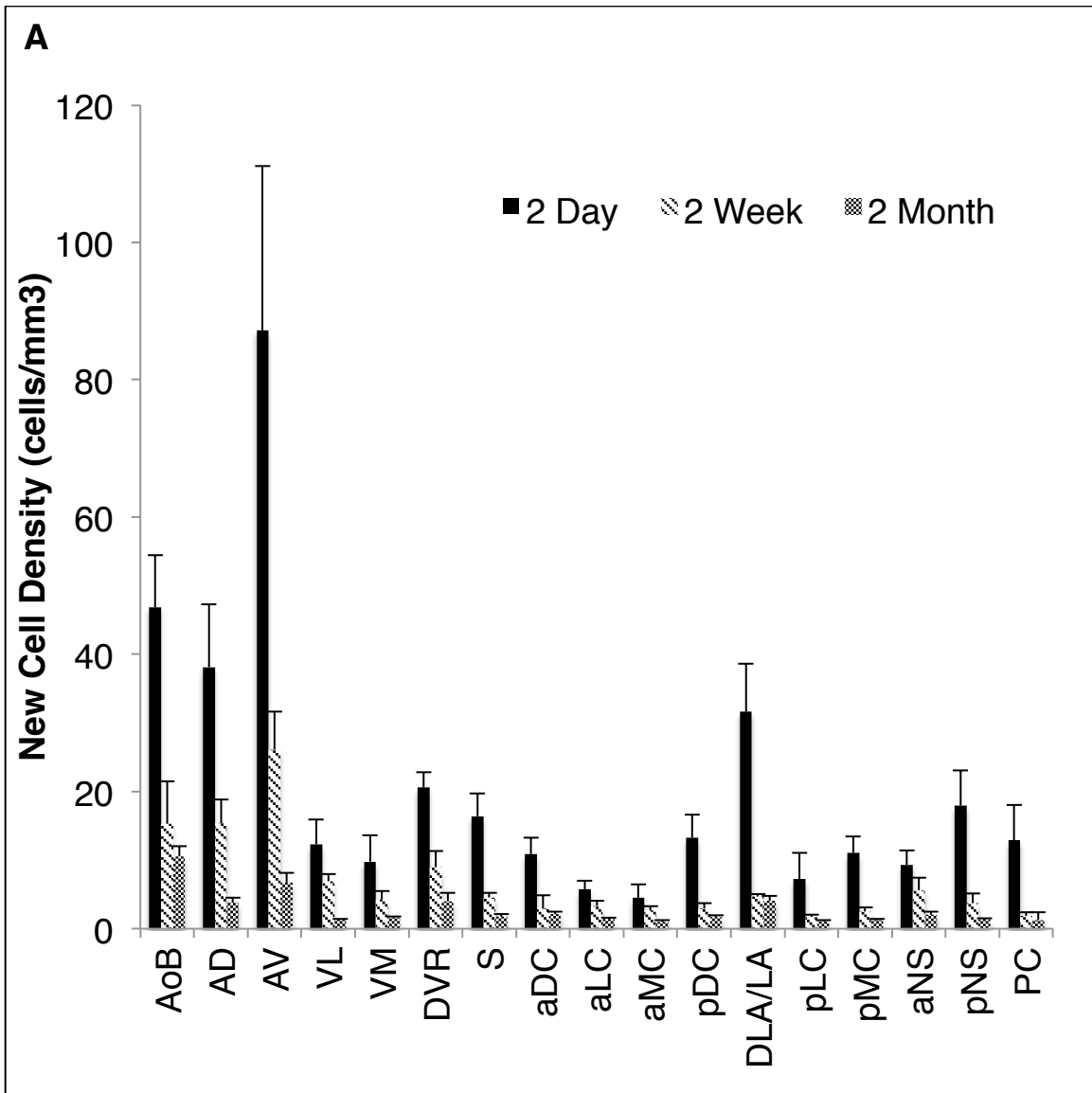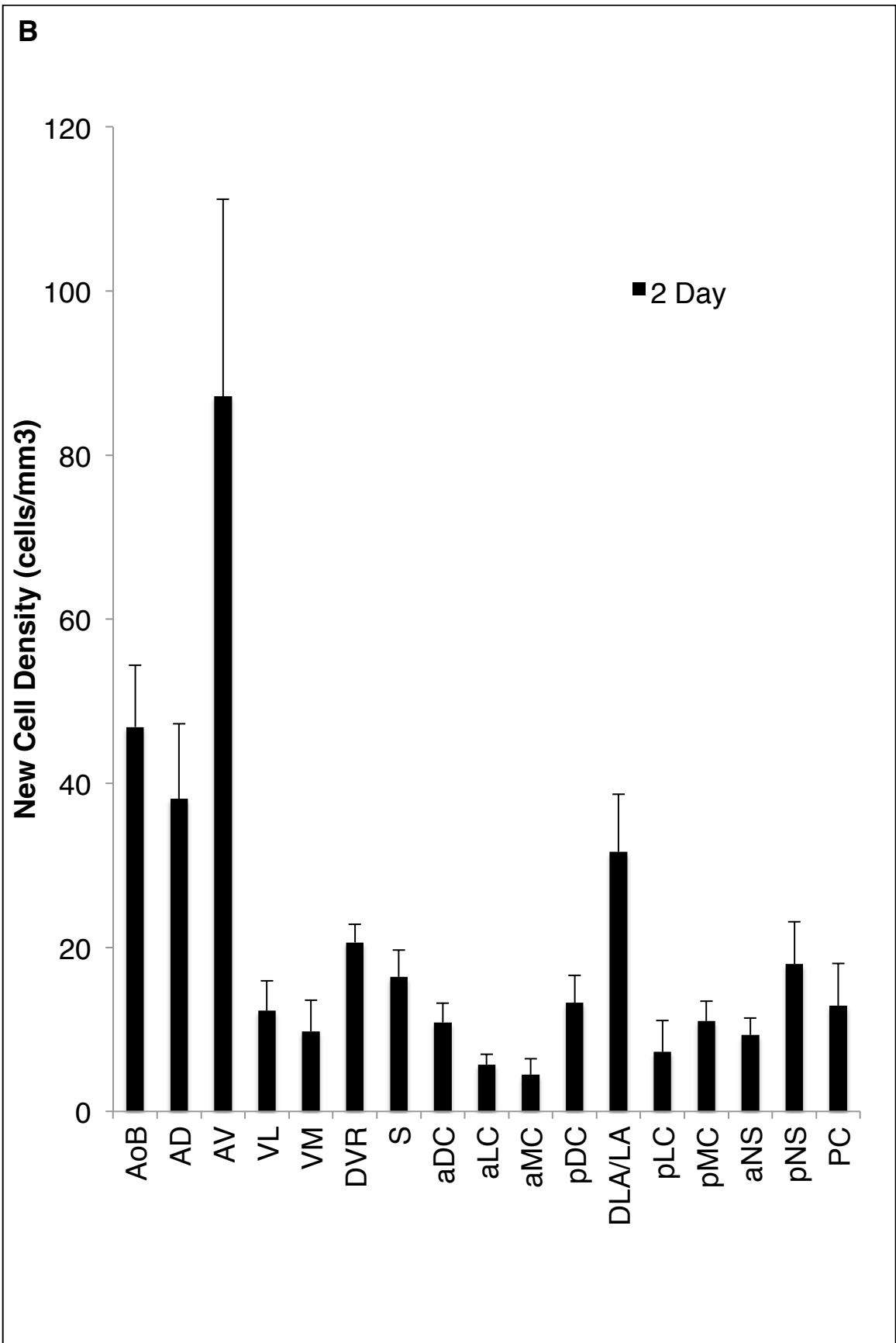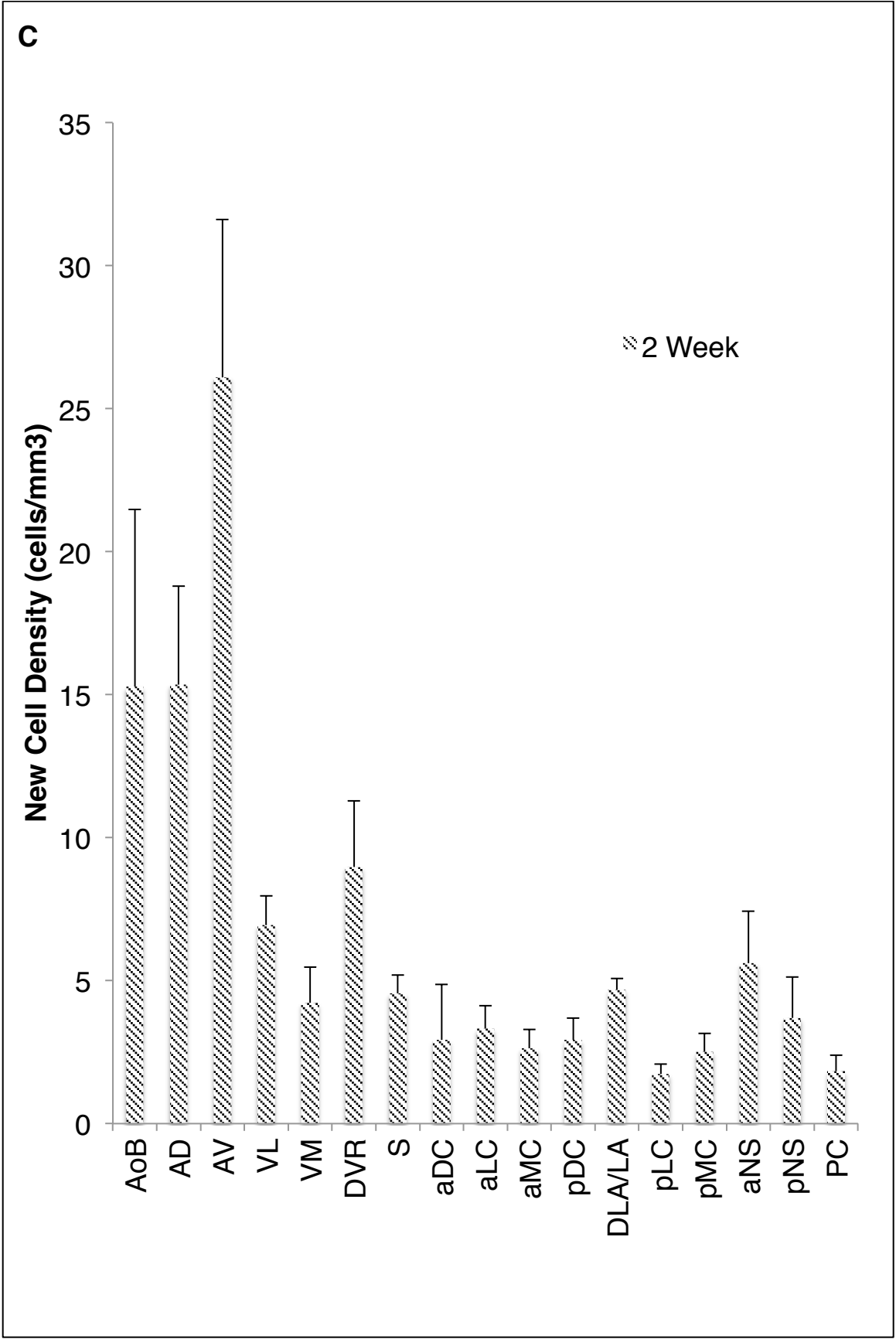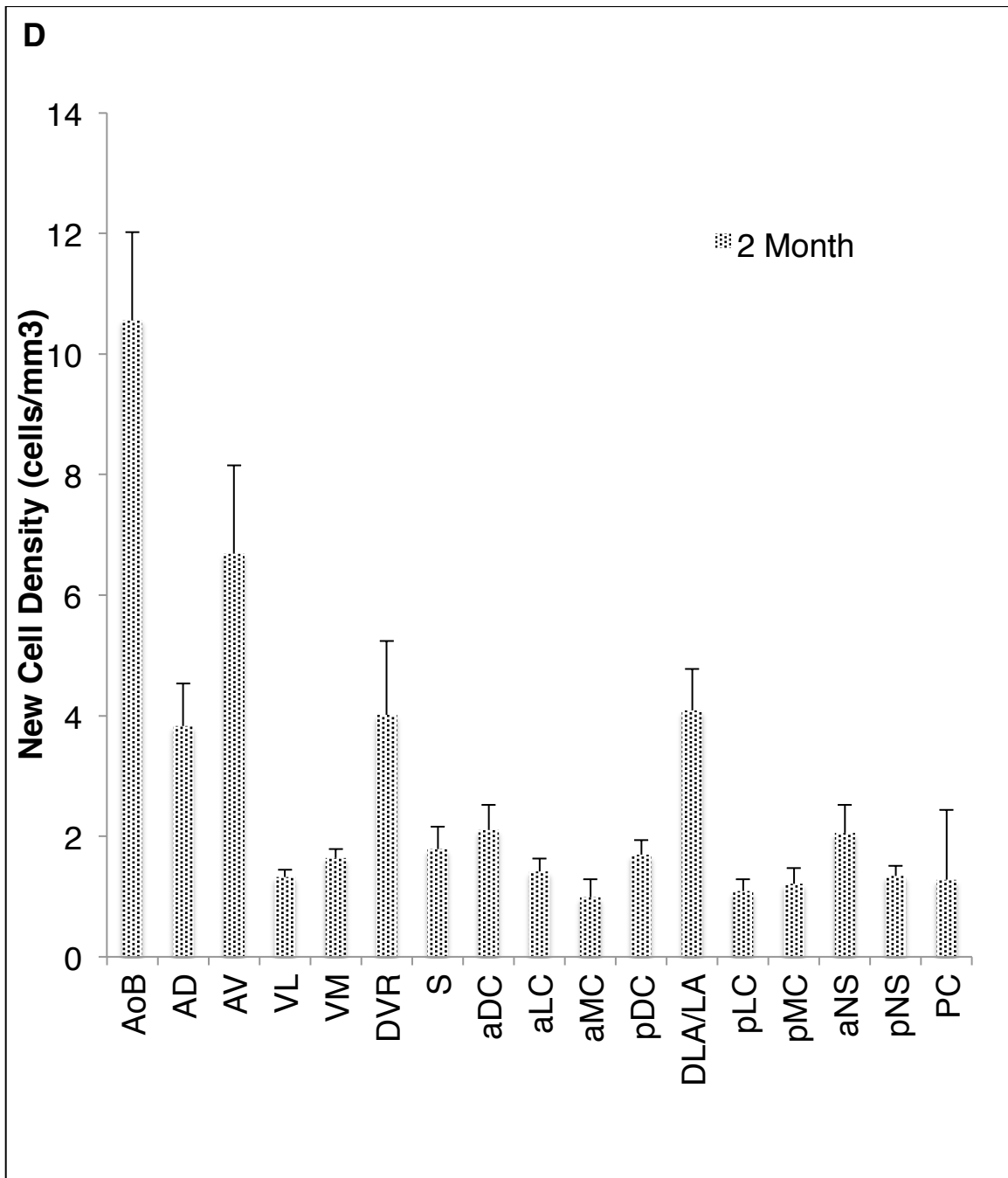
☒ 2 Week

Figure 13: Density of new cells (cells/mm^3; mean ±SE) located in regions of the *P. regius* telencephalon denoted in fig. 10, **A.** 2 days, 2 weeks, and 2 months post-injection. **B.** 2 days post-injection only. **C.** 2 weeks post-injection only. **D.** 2 months post-injection only.
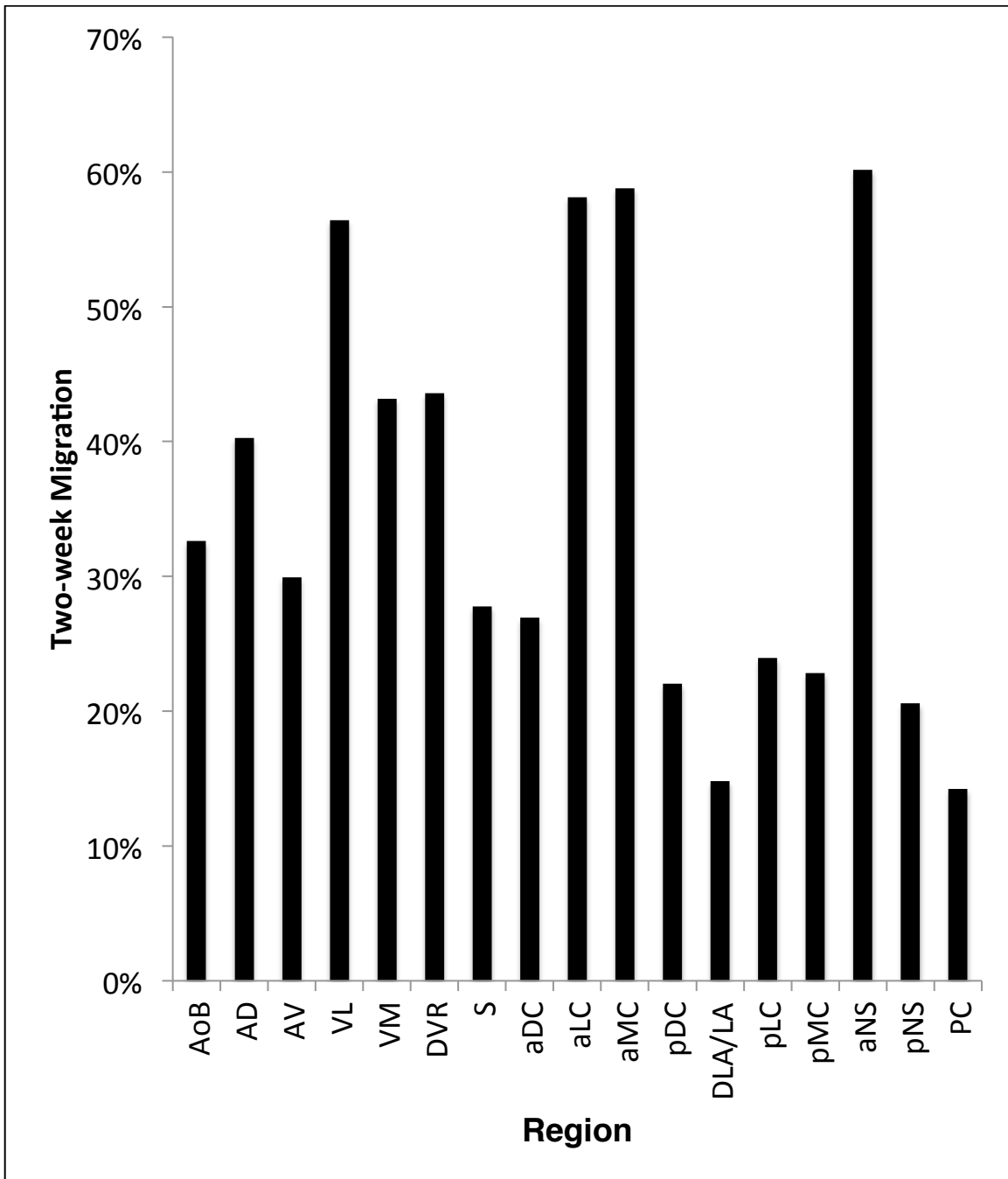
Figure 14: Regional migration of new cells (%cells) determined from [(mean regional parenchymal cell density at 2 weeks)/(mean regional ependymal cell density at 2 days)] in the *P. regius* telencephalon, 2 weeks post-injection.
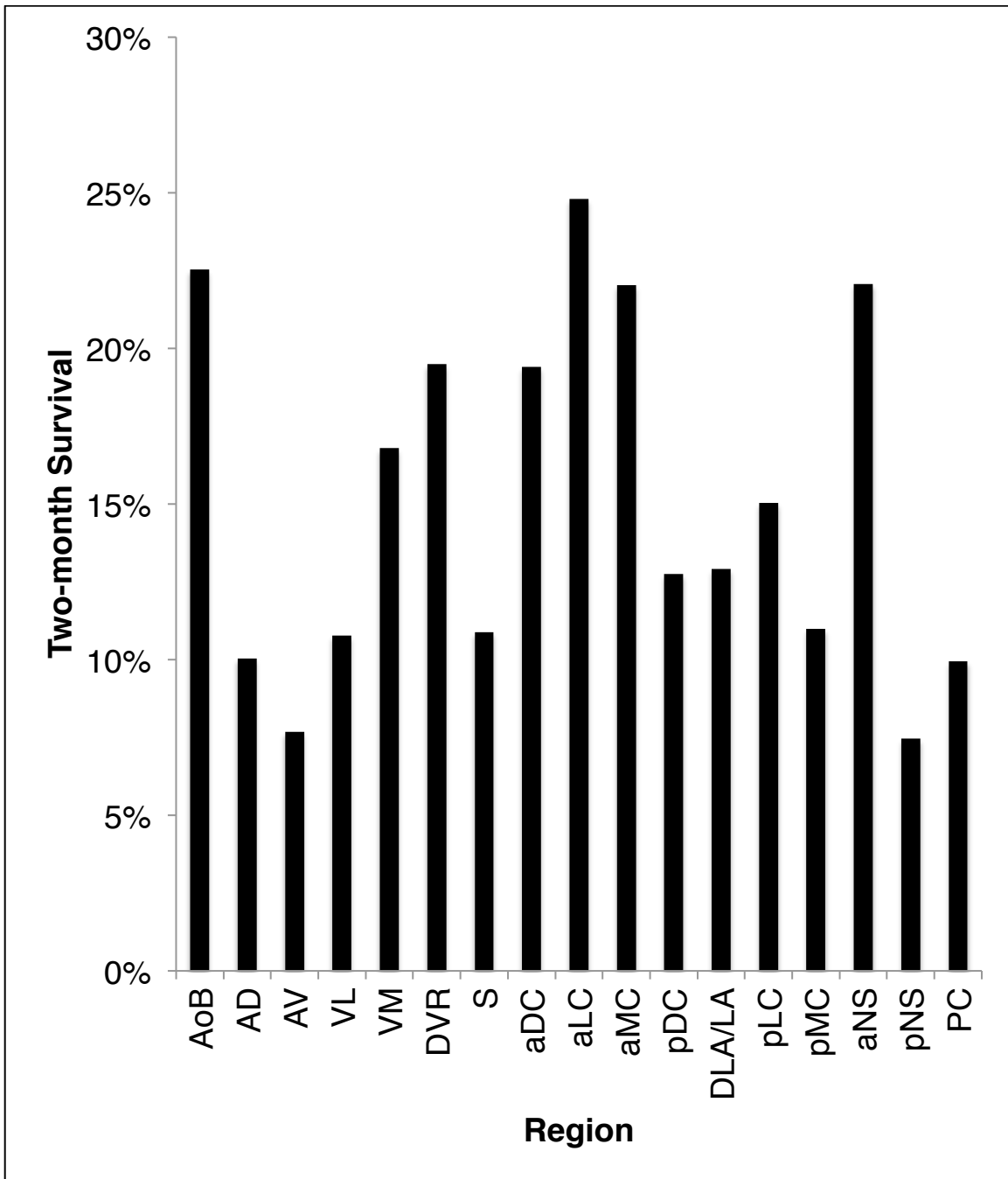
Figure 15: Regional survival of new cells (%cells) determined from [(mean regional parenchymal cell density at 2 months)/(mean regional ependymal cell density at 2 days)] in the P. regius telencephalon, 2 months post-injection.
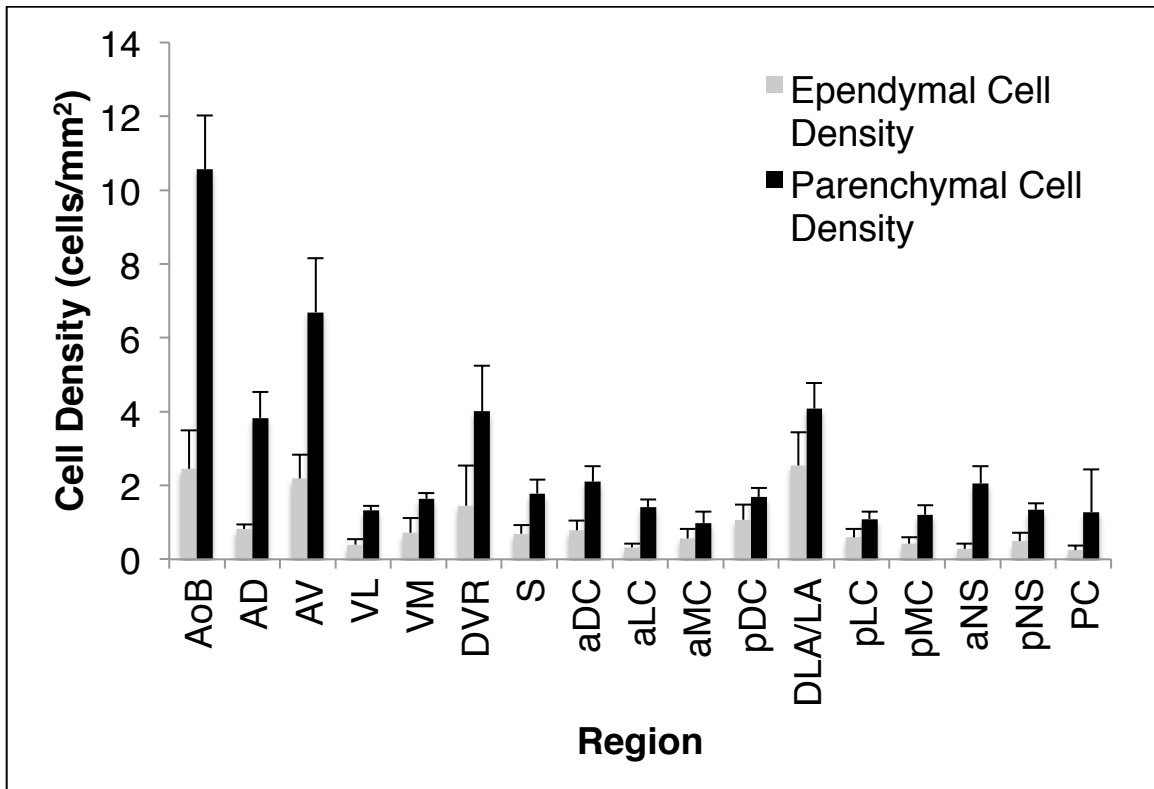
Figure 16: Comparison of cell density in the ependymal zone versus parenchymal zone 2-months post-BrdU injection.

Table 3: Telencephalon regions organized by high or low proliferation combined with high or low migration, and survival.

		Migration		Survival	
		High	**Low**	**High**	**Low**
Proliferation	**High**	AD, AV, DVR, aNS	DLA/LA	AOB, DVR, aNS	AD, AV
	Low	VL, VM, aLC, aMC,	pLC	aLC, aMC	VL

Discussion

Dosage of BrdU

In accordance with previous studies of BrdU dose, we detected an effect of dose on ependymal cell marking. The highest tested dose (250mg/kg) marked a significantly higher density of cells than the lowest tested dose (50mg/kg). This finding supports previous studies of this relationship (Burns and Kuan, 2005; Cameron and Mckay, 2001; Hancock et al., 2009) and demonstrates that dose affects cell marking not only in mammalian species, but also in reptiles. In this experiment, the maximum BrdU dose was limited to 250mg/kg because BrdU precipitated from solutions containing more than 20mg/mL and injecting a large subcutaneous bolus (in excess of 21mL for a 1.2kg snake) is physically challenging, requiring multiple injection sites, which is potentially stressful to the animal. Since previous studies used much smaller animals (<300g), experimenters were able to inject BrdU doses higher than 250mg/kg and determine the optimal dose. I expect doses larger than 250mg/kg would mark significantly more cells than the 100mg/kg dose, yet administration of higher doses would require using a solvent able to provide higher miscibility for BrdU in addition to, or in lieu of, using smaller snakes. While higher doses in a different solution could potentially provide further marking, this may not be necessary, as the 100mg/kg dose yielded BrdU cell density that was not significantly different from the 250mg/kg dose. Larger subcutaneous doses are inadvisable because the buildup of a large bolus creates considerable pressure against the syringe, causing leakage through the injection site post injection. These

large pressures could damage the snakes' tissues, cause BrdU to leak, potentially exposing the administrator to BrdU—a carcinogen—and expose snakes administered larger bolus volumes to less BrdU m/m relative to snakes administered smaller boluses.

Proliferation, migration, and survival

Two days after BrdU injection, cells were labeled throughout the telencephalon along the lateral ventricles, especially at the lateral sulci, indicating areas of proliferating cells. The greatest proliferation took place at the ventricles nearest the AOB, AD, AV, and DVR. Many of these newborn cells migrate to the parenchyma throughout the telencephalon and survive for at least 2 months. On the other hand, some of them continue to stay in the ependymal region and do not migrate even after 2 months. This system of reduced migration across the VZ parallels other squamate studies, and like in other squamate studies (*P. hispanica, T. mauritanica*), differs from turtles, which exhibit even slower migration of proliferating cells across the VZ (Font et al., 2001). The ratio of ependymal-cells-to-parenchymal-cells in the DLA/ LA region and DC at least one month post injection appears to be consistent among all squamates (Font et al., 2001).

There appears to be a dichotomous relationship of neurogenic activity between brain regions involved with more short-term or long-term brain functions. Regions that relay regularly changing olfactory and vomeronasal stimuli as part of the amygdalar system (short-term functions) may require constant growth of new neurons and/or higher cell turnover. The DLA/LA region, which is considered the

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reptilian homologue to the basolateral amygdala, has high proliferation rates but does not seem to have as high of a survival rate. Additionally, in mammals, the regions involved in processing olfactory input (the rostral migratory stream; RMS) have relatively high neurogenic activity (Altman, 1969; Pencea et al., 2001). In reptiles, the homologs to the RMS are likely located in the retrobulbar regions (AD), the AV containing the TUB, and the AOB which relays vomeronasal and olfactory sensory information. These regions also seem to have high proliferation rates, but possibly lower survival.

Conversely, regions known for long-term functions like spatial memory, exploratory behavior and species-specific behavior like territorial displays, exhibited lower proliferation rates, but less attrition and higher survival. Examples of these regions are the MC and DC associated with spatial memory (Baird Day, et al., 2001; Lopez et al., 2003; Holding et al., 2012), the VL containing the STR, associated with species-specific behavior and working memory (Greenberg, 1977; Packard and Knowlton, 2002; Tarr, 1982), and the VM containing the ACC responsible for exploratory behavior and behavioral reinforcement (Olds & Milner, 1954; Distel, 1978; Greenberc et al., 1979; Parent, 1986).

MC and DC: Hippocampal homologues

The reptilian MC and DC are widely regarded as homologous to the mammalian and avian hippocampus (Bruce and Butler, 1984; Butler and Hodos, 1996; Rodriguez et al., 2002, Holding et al., 2012). The small-celled portion of reptilian cortex is considered homologous to the dentate gyrus of the mammalian

hippocampus, and the large-cell portion is considered homologous to the CA fields

of mammals (Treves et al., 2008). No CA-field subdivisions have been distinguished

(Papp et al., 2007), although a system has been proposed for further investigation

(Delgado-González et al., 2008). As in the dentate gyrus of the adult mammalian

hippocampus (Kuhn et al., 1996; van Praag et al., 2002), the MC is associated with

continuous neurogenesis during adulthood in reptiles (Bruce and Butler, 1984; Butler

and Hodos, 1996; Casini et al., 1986; Hoogland et al., 1987, 1998; Lopez-Garcia et

al., 1992, 2002). Integration of new granule cells is essential to hippocampal function

in the mammalian dentate gyrus (Clelland et al., 2009; Dupret et al., 2008; Saxe et

al., 2006; Shors et al., 2001). Recent studies have demonstrated that new granule

cells allow for "pattern separation"—the formation of distinct memory representations

originating from similar information (Sahay et al., 2011a). Pattern separation is the

orthogonalization of input that prevents memories from interfering from one another

(Sahay et al., 2011b). While there is no direct evidence for pattern separation in

reptiles, the two systems are so functionally and structurally similar that a system

similar to CA3 could potentially occur in reptiles and should be investigated.

The basal regions: The striatum and the nucleus accumbens

Very recently, it has become clear that adult neurogenesis takes place in the

human STR, displacing the previous canon that it only takes place in the olfactory

bulb and hippocampus (Kempermann, 2014). Using carbon-14 dating techniques,

Ernst et al. (2014) found that striatal interneurons undergo turnover at a rate of 2.7%

annually, and most impressively, that these neurons are depleted in Huntington's

disease patients. Other mammalian studies have supported these findings

(Arvidsson et al., 2002; Dayer et al., 2005; Luzzati et al., 2006; Yamashita et al.,

2006), but none of them until now showed complete cell proliferation, neurogenesis,

and functional integration in an undamaged brain (Bonfanti and Peretto, 2011).

For a couple decades, there has been limited evidence for adult neurogenesis

in the STR of lizards (Penafiel et al., 2001; Pérez-Cañellas and García-Verdugo,

1996) and turtles (Pérez-Cañellas et al., 1997). When Pérez-Cañellas and García-

Verdugo (1996) observed BrdU-labelled brain tissue from *T. mauritanica*, they saw

very few cells in the STR and high variability among individual study animals. The

cells did not concentrate in any specific area and instead were uniformly distributed.

The new cells also appeared to move both radially and tangentially, instead of

following the radial processes of radial glial cells indicating migration similar to the

RMS system in mammals. In the current study, the high proliferation rate and low 2-

month survival within the STR may be indicating a similar migration. New cells may

be proliferating at the ventricular ependymal layer, migrating out to the parenchyma,

and then continuing to travel rostrally out of the STR to other brain regions. Sagittal

sectioning could show whether cells are moving rostrally in the STR as well as other

regions.

There has been little literature about adult neurogenesis in the ACC, likely

because evidence is lacking that it occurs there in mammals. In reptiles, migrating

neurons have been found in ACC of *P. hispanica,* as evidenced by the neuronal

marker PSA-NCAM (Ramirez-Castillejo et al., 2002a), but whether the cells were of

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proliferative origin has not been confirmed. In amphibians, Almli and Wilczynski

(2007) found cell proliferation and survival after 30 days in the ACC of *Hyla cinerea*

(American green tree frog). It has been further supported in *Lithobates catesbeianus*

(American bullfrog) and *Xenopus laevis* (African clawed frog), but the final

phenotype of these cells is yet to be determined (D'Amico et al., 2011; Simmons et

al., 2008). In mammals, the ACC has been implicated as responsible for reward-

seeking behavior and motivation (Cador et al., 1989; Knutson et al., 2001), including

addiction-based behavior (Hoebel et al., 1983). While it is likely that there is a link

between adult neurogenesis in the ACC, sensory systems, and reinforcement based

on what is perceived, there are so few data regarding adult neurogenesis in the ACC

that it is too early to make any hypotheses as to what is taking place in this system.

Amygdaloid regions

The mammalian amygdala is a site where modulation and memory consolidation

occurs in response to emotional stimuli such as fear (Martínez-Garcıa et al., 2009) or

during reward processes (Stuber et al., 2011). However, the amygdala itself is not a

memory storage site (Malin and McGaugh, 2006). It is also important in processing

information about pheromones and other olfactory or vomeronasal input, causing it

to directly influence the endocrine system (Halpern and Martínez-Marcos, 2003) and

behavior during confrontations with conspecifics (agonistic behavior) (Meredith and

Westberry, 2004). Support for mammalian amygdalar neurogenesis has been limited

to studies indicating proliferation, cell genesis, and specification in *Orictolagus*

cuniculus (New Zealand white rabbits) (Luzzati et al., 2006), *Mus musculus*

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(laboratory mice) (Shapiro et al., 2009), and *Saimiri sciureus* (common squirrel monkeys) and *Macaca fascicularis* (crab-eating macaques) (Bernier et al., 2002). In the present study I have found the reptilian DLA/LA is a region of high proliferation, but low migration and survival, which coincides with its role, not in storage, but in encoding sensory memory.

Olfaction and the RMS

The generalized AD as defined in this paper likely contains the rostral migratory stream (RMS). In mammals, the RMS is well known as a highly neurogenerative and neuromigratory region, and has been given the name, "Rostral Migratory Stream," because it is where neuronal precursors migrate from the ependymal zone to the olfactory bulb. The findings of this study confirm studies in other reptiles (Peñafiel et al., 1996; Pérez-Cañellas et al., 1997), indicating the presence of a RMS similar to that of fish (Grandel et al., 2006) and mammals (Pencea et al., 2001). There is no evidence indicating an RMS in amphibians or humans, and limited evidence in birds (Kaslin et al., 2008). These anatomical differences are likely related to differences in the importance of olfaction and/or pheromone signaling in different taxa.

The RMS system could also be responsible for what appears to be higher proliferation and lower parenchymal survival of cells in the retrobulbar regions compared to the more caudal telencephalon. Cells could be surviving at 2 weeks and 2 months and then migrate rostrally out of the study region and into the olfactory bulbs so they are not counted as surviving cells. For example, the RMS cells migrate

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in mice at an average rate of 30μm/hr (Lois and Alvarez-Buylla, 1994). At that rate they could completely migrate out of the study area within 2 months. Further study using sagittal instead of transverse sections could clarify whether RMS migration or attrition is responsible for this result.

DVR

The most prominent pallial structure in reptiles is the DVR (Holmgren, 1925, Northucutt, 1981, Ulinski, 1983). It appears to exhibit not only high proliferation, but also high relative migration at 2 weeks and survival at 2 months. This is not surprising because the DVR has been implicated as a site for processing of sensory information, association, and output for modulation of behavior. A region essential to so many functions likely requires neurogeneration and long-term cellular turnover throughout adulthood in order to provide plasticity for behaviors in response to environmental changes.

Several previous studies in reptiles have found evidence of DVR cell genesis (Delgado-Gonzalez et al., 2011; Font et al., 2001; Marchioro et al., 2005). The mammalian homologue to the reptilian DVR may be the neocortex, claustroamygdaloid regions, or a combination of both; however, the debate over this homology has elicited controversy in the field of evolutionary biology (Bruce and Neary, 1995; Karten, 1997; Molnár and Butler, 2002; Striedter, 1997). Adult neurogenesis in mammals does not occur in the neocortex (Bhardwaj et al., 2006), but there has been evidence of proliferation and cell specification (without identifying neurogenesis as such) in both the amygdala and piriform cortex of rodents and

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primates (Bernier et al., 2002; Bonfanti and Peretto, 2011; Pencea et al., 2001; Shapiro et al., 2007). Thus, if the function of the amygdala and piriform cortex relies on neurogenesis in both mammals and reptiles, the fact that the DVR does exhibit adult neurogenesis may provide further support for a homology with the mammalian amygdala/piriform cortex.

Comparing reptiles to birds, it is likely the nidopallium is the avian homolog to the reptilian DVR (Bruce, 2009). The nidopallium also receives new neurons during adulthood. The caudal nidopallium (nidopallium caudale, "NC") stores song-specific characteristics for later recall, and has been found to be activated in canaries and zebra finches when they are exposed to songs of conspecifics (Bolhuis and Gahr, 2006). Hormones also appear to influence neurogenesis in the nidopallium of songbirds. Testosterone triggers and modulates seasonal surges in neuro- and angiogenesis (Chen et al., 2013). Estrogen receptors and telencephalic aromatase are densely distributed in the NC and they, in conjunction, influence cell survival (Gahr et al., 1993; Shen et al., 1995). Whether or not similar systems exist in reptiles is yet to be determined. The scarcity of studies investigating the roles of hormones in adult neurogenesis in reptiles leaves room for further investigation. The DVR could also potentially be a site where seasonal changes in neurogenesis correspond with pheromones and other cues in reptiles instead of auditory cues as in birds. While studies have investigated the role of pheromones in neurogenesis in other regions (Font et al., 2012), or changes in neurogenesis in the DVR in response to temperature and season (Delgado-Gonzalez et al., 2011; Delgado-González et al.,

2008; Penafiel et al., 2001), no studies thus far have investigated how seasonal pheromonal and hormonal changes affect DVR neurogenesis in adult reptiles.

Olfactory and chemosensory processing

In reptiles, olfactory information enters the brain through the main olfactory bulb (MOB) and is relayed to the lateral (pyriform) cortex (LC). Vomeronasal information, on the other hand, enters through the accessory olfactory bulb (AOB), which transmits to the nucleus sphericus (NS) (Lohman and Smeets, 1993; Lanuza and Halpern, 1997). Due to the importance of pheromone perception, the NS is very well developed in snakes and occupies almost the entire caudal half of the subcortical telencephalon (Schwenk, 1993; Halpern, 1980; Lanuza & Halpern, 1997). The aLC is most likely involved in the convergence of chemosensory and olfactory processing, with projections to the MC for spatial association and memory (Lanuza & Halpern, 1997).

The vomeronasal-AOB pathway terminates at the NS in squamates, and the NS appears to control the vomeronasal system, so that NS neurogenesis should be directly related to neuronal turnover in the vomeronasal epithelium and may affect motor function in response to chemoreception (Lohman and Smeets, 1993; Lanuza and Halpern, 1997; Distel, 1978; Simon, 1983, Perez-Sanchez et al., 1989).

The NS of reptiles in total is considered homologous to the posterior medial cortical nucleus of the amygdala in rodents—a secondary vomeronasal center that is part of the vomeronasal amygdala (Gutiérrez-Castellanos et al., 2014; Martinez-García et al., 2002). The posterior cortical nucleus of the amygdala in female prairie

(*Microtus ochrogaster*) and meadow (*M. pennsylvanicus*) voles expresses adult

neurogenesis colocalized with estrogen receptor-α (Fowler et al., 2005), and

neurogenesis increases in response to exposure to conspecific males (Fowler et al.,

2002). It is likely that the neurogenesis in this region in mammals corresponds to

pheromonal and olfactory input for two reasons: (a) the amygdala receives direct

input from the olfactory bulb, and (b) there is some evidence that exposure to male

bedding alone also elicits an effect (Fowler et al., 2008). In *P. hispanica,* the NS

exhibits the highest rate of incorporation of new neurons (Font et al., 2001). There

have been no studies of neurogenesis in the NS of reptiles in response to

pheromonal input, but Delgado-González et al. (2008) found there to be seasonal

plasticity, indicating pheromonal changes could potentially be associated with NS

neurogenesis.

The development of the NS is clearly related to chemosensory function and

ecology (Halpern and Martínez-Marcos, 2003). Balaban (1978) studied *P. s.

elegans*, which spend considerable time underwater and are thus not exposed to

consistent olfactants. He found that they completely lack the NS region, which may

be further emphasizing olfactary- versus non-olfactory-oriented evolutionary

development. The NS may undergo neurogenesis to provide a new population of

cells that can process new chemosensory signals as *P. regius* changes

environments. These data indicate further experimentation is necessary to determine

how pheromones and environmental changes affect NS neuroplasticity.

The septum

Font et al. (1998b) posited that the lateral septum is involved in the expression of appetitive behaviors (e.g. food and water intake), aggressive/defensive behavior, and reproductive physiology in reptiles, and may be considered a "distributor of behaviors" (Distel, 1978; Krohmer and Crews, 1987a; Tarr, 1977). The septum may mediate social memory including relating olfactory cues to parental behavior, intraspecific aggression, dominant-subordinate relationships, or defensive/agonistic behaviors and territoriality; which is why it is considered key to the social behavior network (Dantzer et al., 1988; Lanuza and Martínez-García, 2009; Numan, 2000; Sheehan and Numan, 2000; Yang and Wilczynski, 2007). In mammals, the main afferent to the lateral septum is a projection from the hippocampal formation (Sheehan et al., 2004), which provides spatial and non-spatial contextual information. Hence the lateral and medial septum are likely responsible for territorial motivation—behaviors in response to contextual cues (Newman, 1999). In *P. regius,* the septum appears to undergo proliferation at rates consistent with the rest of the telencephalon, albeit lower than average. This proliferation rate differs from *Trachemys scripta, Podarcis hispanica*, and *T. mauritanica,* for which septal proliferation is either exceptionally low or nonexistent (Perez-Cañellas et al., 1997; Perez-Cañellas and Garcia-Verdugo, 1996; Font et al., 1997).

Rostral-caudal effects

Cell migration was also considerably lower in the caudalmost portions of the telencephalon (DLA/LA, PC, pMC, pDC, pLC)—a trend that is best illustrated by the contrast between the aNS and pNS. Almost 60% of cells in the aNS migrated to the parenchyma after two weeks, yet only ~ 21% of pNS cells entered the parenchyma over the same period, indicating the aNS has almost three times the migration rate of the pNS. This rostral to caudal gradient was also found by Holtzman and Halpern (1991) in embryonic *Thamnophis* spp. This might occur because the caudal portion has higher proximity to the rest of the telencephalon and thus plays a larger systemic role in olfaction and reinforcement with other systems.

There was also a contrast between the aMC and pMC. Proportional migration after 2 weeks is approximately 2.5x higher in the aMC than the pMC, and proportional survival after 2 months is approximately 2x higher. This rostral-caudal trend is also strong in the LC wherein the difference between caudal and rostral is largest after 2 weeks (approximately 2.4x rostral-caudal). This trend is weaker in the DC (approximately 1.5x rostral-caudal). This coincides with other reptile studies that have found caudal-rostral differences in the MC. Hoogland et al. (1994) argued that, in contrast to ground-dwelling geckos, *Gekko gecko* (tokay geckos) have a medial cortex that is hodologically divided into two separate regions, one rostral and one caudal, which better enables them to spatially navigate walls and ceilings in 3-dimensional space. Since both climbing trees and burrowing underground in 3-dimensions is part of the behavioral repertoire of *P. regius*, they too may require a

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MC system that allows for two interconnected but structurally and functionally different MC systems (Hoogland and Vermeulen-Vanderzee, 1995). Regional variation in the MC of snakes is further supported by Roth et al. (2006), who found sex differences in cortex volume relative to telencephalon volume only in the caudal half of the medial cortex.

Conclusions

This study is intended to serve as a preliminary investigation of proliferation, migration, and survival of newly born cells in pythons. BrdU administration was dose-dependent as in mammals, and it was determined that the most appropriate dose for BrdU administration is 100mg/kg when it is dissolved in saline solution. When measuring the proliferation, migration, and survival of new cells in several telencephalic regions, some regions exhibited high proliferation, with low relative migration and survival (and for other regions the result was vice-versa). These trends may be due to differences in regional function. Regions involved in short-term or spontaneous functions like olfactory processing may require more neurogenesis in response to rapidly changing environments, while regions with long-term functions like spatial memory may require lower cell turnover as cellular perpetuity becomes a higher priority.

Contrary to what was predicted based on trends among squamate taxa, there was relatively little proliferation in the MC. As predicted, however, there was relatively high proliferation in the AOB, DVR, and NS. Also as predicted, there was relatively high migration/survival in MC, AOB, DVR, and NS. This further clarifies the

regional differences in proliferation and survival of brain cells throughout squamate reptiles.

Cell types were not distinguished using immunohistochemical markers, stains or other techniques. To date, no published studies have successfully used neuronal markers outside of the posterior DVR in adult snakes, and glial markers have limited reliability (Nomura et al., 2013; personal observation). Future studies should focus on determining the phenotype of BrdU marked cells (as neurons or glial cells), however it may require further steps in developing markers for use in adult snake brain tissue because antibodies for neuronal markers (DCX, Tuj1, NeuN) refined for use in mammals have been unsuccessful in labeling cells in snake brain tissue in pilot studies (personal observation).

Additionally, determining which genes and proteins are responsible for developmental differences can reveal aspects of human evolution. For example, Aboitiz's (2011) comparative investigation has suggested Wnt signaling has contributed to substantial elaboration of the dorsal pallium in mammals, while Pax6 upregulation is responsible for the expansion of the ventral pallium in non-mammalian amniotes. Further functional studies determining which proteins are responsible for the upregulation of neurogenesis and neuroregeneration are especially important because they could provide insight into possible therapies for neural diseases.

Generalized regions were used for calculating cell density in this study, which provides less specific data for analysis. Regions like the VM that have smaller nuclei

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(subregions) could potentially have very high density of new cells in some of these nuclei. But this density could potentially go undetected because only the entire region was investigated and not the subregions. Future studies should apply stains for distinguishing smaller brain nuclei and determine whether cells are migrating to those specific nuclei within the larger regions defined in this study.

Finally, it is likely not coincidental that regions expected to be contributing to olfaction and basal-amygdalar function (ACC, STR, DLA/LA, and LC) have highest proximity to the sulci of the ventricles of snakes. I expect this occurs because cells tend to proliferate and migrate outward from "hot spots," which allows organisms like *P. regius* that rely on discrimination of olfactory stimuli to have a continuous supply of neurons to aid in processing of odors and associating them with positive or negative stimuli in new environments. This hypothesis is contrary to the findings of Font et al. (2001) in other reptiles, and may be limited only to snakes. This proposition, along with many of the other hypotheses stated heretofore, may be further refuted or supported by additional studies in a wider range of snake species.

Snakes exhibit several unique characteristics, including limbless body structures, infrared sensory systems, and specialized pheromonal sensory systems that can provide new insights into evolutionary physiology. It is of great benefit to neurophysiology that a wide range of snake species be further investigated to determine trends that can better uncover systems underlying neurogenesis and neuroplasticity in an evolutionary context.

Abbreviations

ACC	Nucleus Accumbens
ACH	Acetylcholine
AD	Anterior Dorsal Region
ADVR	Anterior Dorsal Ventricular Ridge
aDC	Anterior Dorsal Cortex
aLC	Anterior Lateral Cortex
aMC	Anterior Medial Cortex
aNS	Anterior Nucleus Sphericus
AOB	Acessory Olfactory Bulb
AV	Anterior Ventral Region
BrdU	5-bromo-2'-deoxyuridine
BrdU-ir	BrdU-immunoreactive
D	Diencephalon
DC	Dorsal Cortex
DLA/LA	Dorsal Lateral Amygdala/Lateral Amygdala Region
DVR	Dorsal Ventricular Ridge
GABA	γ-Aminobutyric acid
LC	Lateral Cortex
MB	Midbrain
MC	Medial Cortex
NS	Nucleus Sphericus
OB	Olfactory Bulb
OT	Optic Tectum
P	Pineal Gland
PC	Posterior Cortex
PDVR	Posterior Dorsal Ventricular Ridge
pDC	Posterior Dorsal Cortex
pLC	Posterior Lateral Cortex
pMC	Posterior Medial Cortex
pNS	Posterior Nucleus Sphericus
RMS	Rostral Migratory Stream
S	Septum
STR	Striatum
TUB	Olfactory Tubercle
VL	Ventral Lateral Region
VM	Ventral Medial Region

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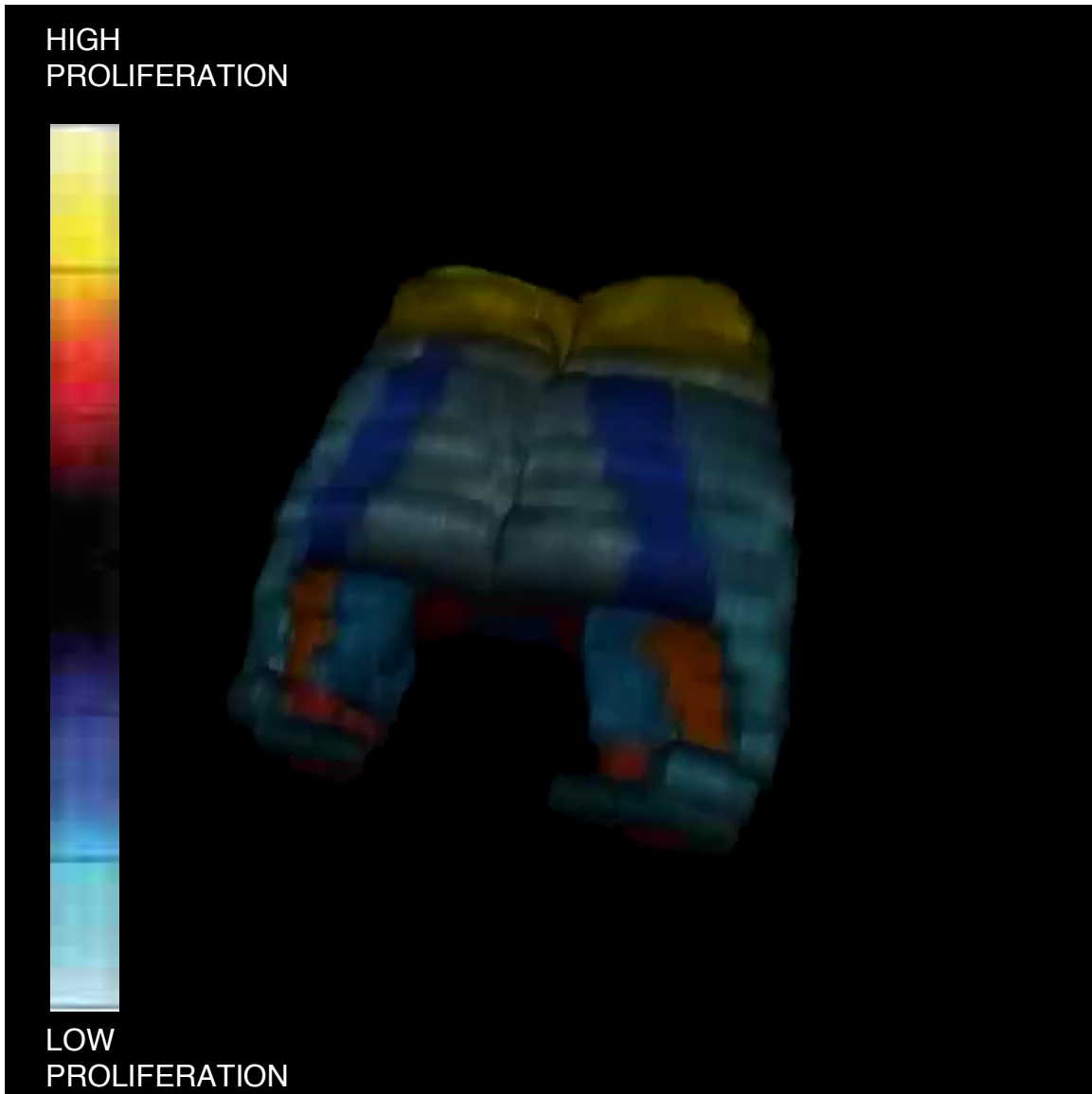
Appendix

Figure 17: Heat map representing proliferation (2 days) in the telencephalon of *P. regius*. Hot colors (yellow, orange, red) represent areas of high proliferation, while cold colors (purple, blue, light blue) represent areas of low proliferation. The rostral end and caudal end are topmost and bottommost, respectively. 3D video can be found at http://youtu.be/zMj8d42o3_4.
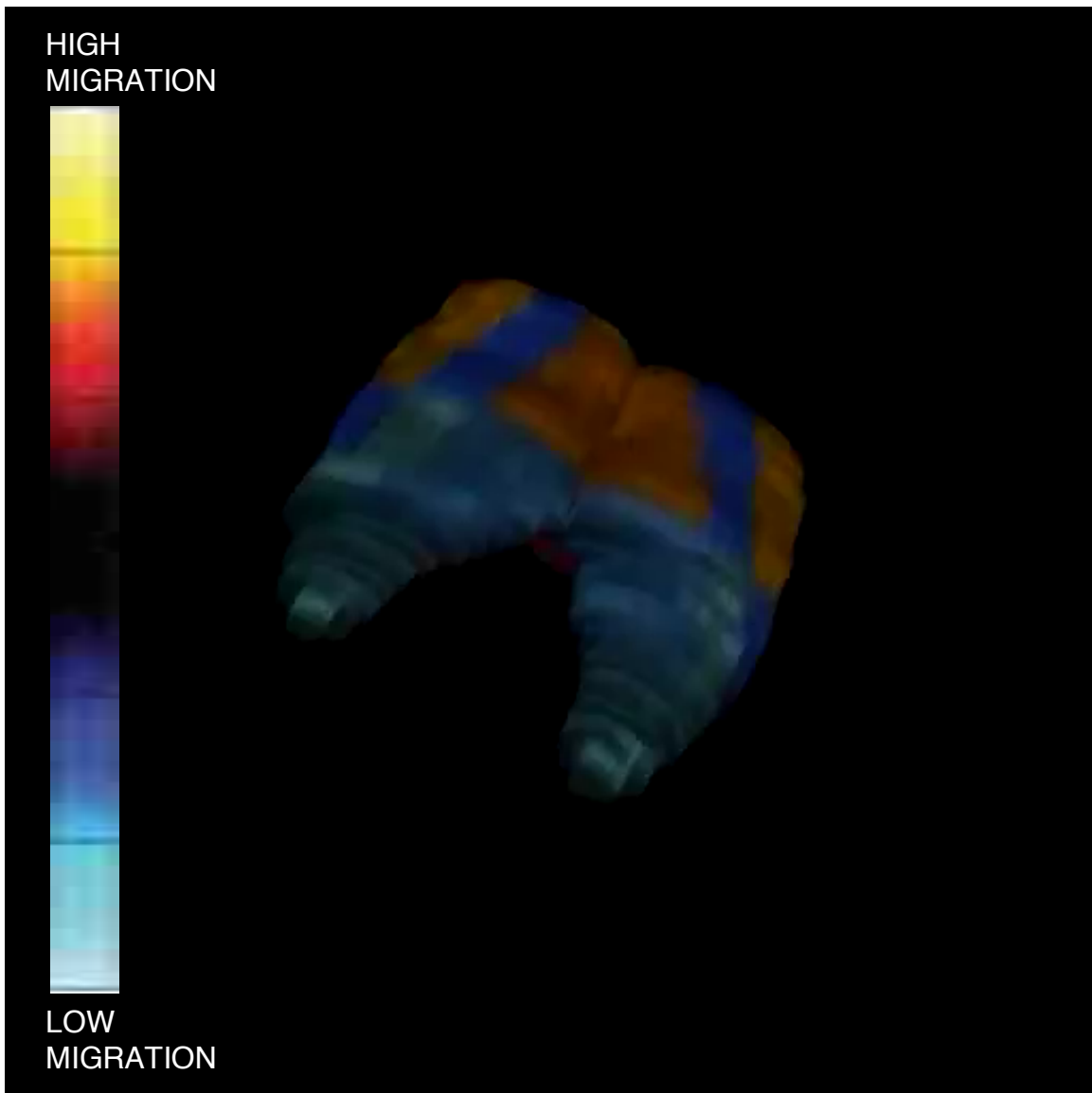
Figure 18: Heat map representing migration (2 weeks) in the telencephalon of *P. regius*. Hot colors (yellow, orange, red) represent areas of high migration, while cold colors (purple, blue, light blue) represent areas of low migration. The rostral end and caudal end are topmost and bottommost, respectively. 3D video can be found at http://youtu.be/QZXKkCTCHsM.
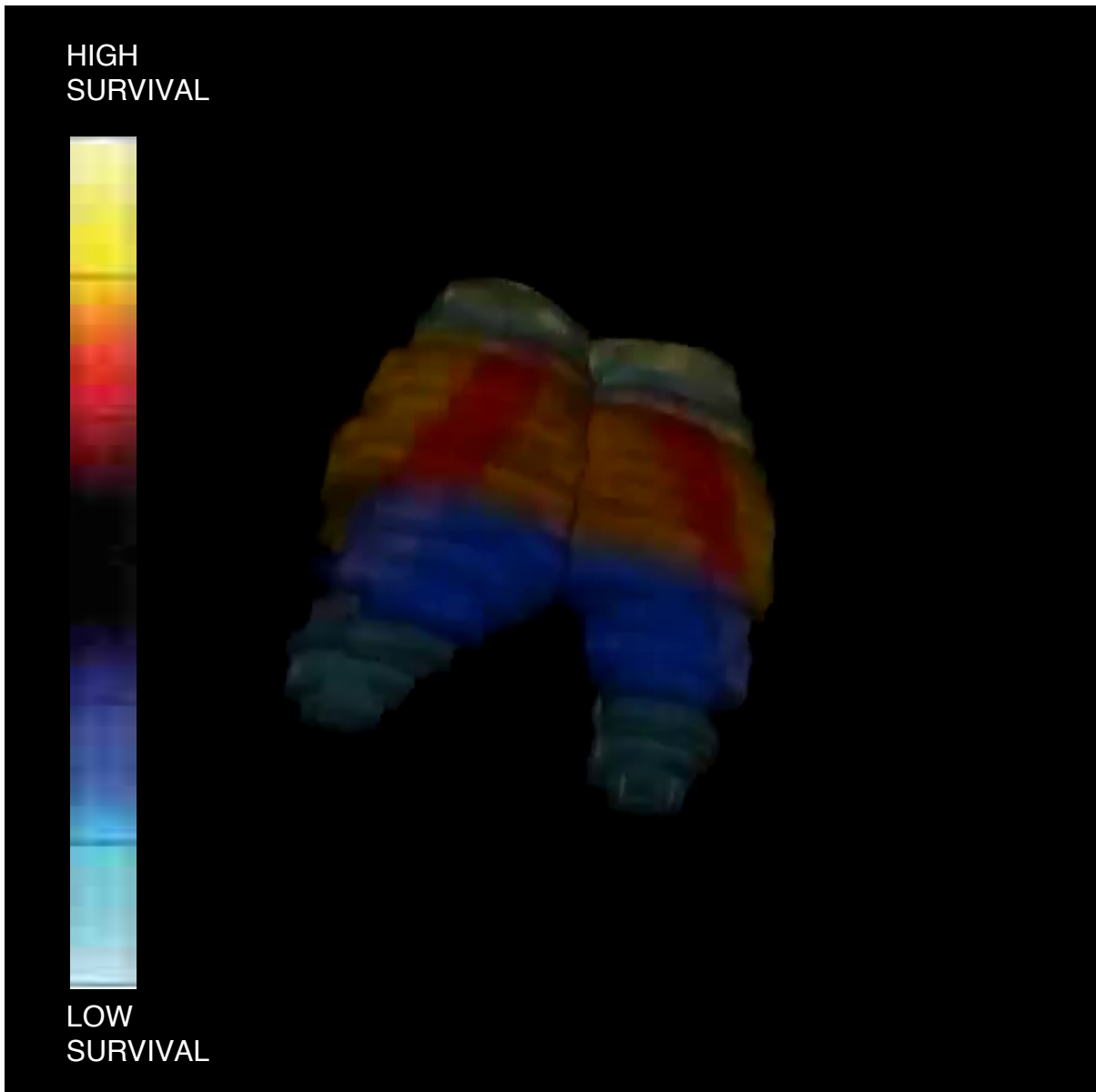
Figure 19: Heat map representing survival (2 months) in the telencephalon of *P. regius.* Hot colors (yellow, orange, red) represent areas of high proliferation, while cold colors (purple, blue, light blue) represent areas of low proliferation. The rostral end and caudal end are topmost and bottommost, respectively. 3D video can be found at http://youtu.be/usnQ5F0AQJk.