Dopaminergic Input to and Reception in the Auditory Midbrain

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ABSTRACT

In anurans acoustic communication mediates reproductive behavior and fitness, making the auditory system a robust model for studying how biologically relevant sounds are processed. Neuromodulators, such as dopamine, can significantly alter auditory neural processing via modulation of action potential initiation and regulation of response properties in several species such as mice, songbirds and fish. Recently, dopamine has been shown to modulate neuronal response properties in the torus semicircularis (torus) of bullfrogs (*Rana catesbeiana)*. The torus is the anuran homolog of the mammalian inferior colliculus. Because the torus receives input from all ascending and descending auditory processing centers and is known to play a crucial role in the analysis of behaviorally relevant sounds, sources of dopamine input and which receptors receive it are of interest. To this end, a precursor in the dopamine synthesis pathway, tyrosine hydroxylase, and dopamine receptors were visualized within the bullfrog brain via immunohistochemistry. Results indicate a potential of seven dopaminergic sources to the torus suprachiasmatic nucleus, dorsal hypothalamus, ventromedial thalamic nucleus, posterior thalamic nucleus, central thalamic nucleus, anteroventral tegmental nucleus, and the ventromedial border of the solitary. The presence of axonal tracts and synaptic boutons positive for tyrosine hydroxylase within all nuclei of the torus indicate dopamine transmission to and reception the torus. The dopamine is received by dopamine 2 receptors in all subnuclei of the torus. These findings fill critical gaps in our knowledge about the neural basis of signal recognition and the role of dopamine in this process.

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ABBREVIATIONS

A – anterior thalamic nucleus
Av – anteroventral tegmental nucleus
C – central thalamic nucleus
D1R - dopamine 1 receptor
D2R - dopamine 2 receptor
DA – dopamine
DMN – dorsal medullary nucleus
Hyp - hypothalamus
MOT – motor nuclei
NLL – nucleus of lateral lemniscus
P – posterior thalamic nucleus
Pm – pallium
PoA – preoptic area
Prv – pretrigeminal nucleus
SC – suprachiasmatic nucleus
Sep – septal complex
SON – superior olivary nucleus
Str - striatum
TH - tyrosine hydroxylase
Tl - laminar nucleus of the torus
Tm - magnocellular nucleus of the torus
Tp - principle nucleus of the torus
Torus - torus semicircularis
VM – ventromedial thalamic nucleus

Specific to Figure 2:
Olf Bulb - olfactory bulb
Tel - telencephalon
Dien - diencephalon
Tec - optic tectum
Cbl -cerebellum
Med - medulla
N I - olfactory nerve
N II - optic nerve
N V - trigeminal nerve
N VIII - acousticovestibular nerve

1. INTRODUCTION

Acoustic communication mediates reproductive behavior in many animal species, including anurans (frogs and toads), thereby contributing to their overall biological fitness. The neural mechanisms mediating acoustic communication in these species are, therefore, of great interest. Owing to the wealth of information regarding the role of sound in their reproductive behavior, as well as the structure and function of their auditory system, anurans have become a robust model for studying how biologically relevant sounds are processed in the nervous system (Wells and Schwartz 1984).

Neuromodulators, such as dopamine (DA), can significantly alter auditory processing via modulation of action potential initiation and regulation of neuronal response properties to sounds in several species such as mice, songbirds and fish (Forlano et al. 2014 and Leblois et al. 2010). In frogs, DA has also been shown to modulate the auditory responses properties of neurons in the torus semicircularis (torus) (Hall et al. 2016). The torus is an important midbrain auditory center receiving convergent input from auditory structures throughout the brain as well as audiomotor pathways. However, the source of dopaminergic input to the auditory midbrain and identity of the receptors receiving it are currently unknown. To this end, immunohistochemistry was utilized to localize and visualize dopaminergic cells throughout the brain via antibodies to the DA precursor, tyrosine hydroxylase (TH). DA receptors were identified within the torus by immunohistochemistry for DA 1 and 2 receptors (D1R and D2R, respectively).

TH positive somata were found in 7 structures throughout the brain, 6 of which provide descending projections to the toral nuclei, and 1 of which provides ascending input to the torus. The suprachiasmatic nucleus, dorsal hypothalamus, ventromedial thalamic nucleus, posterior thalamic nucleus, central thalamic nucleus, anteroventral tegmental nucleus project cadually; and the ventromedial border of the solitary projects rostrally to the torus. Any region or combination thereof could be providing dopaminergic input to the 3 nuclei of the torus. Within the torus, axonal tracts and synaptic boutons contain TH indicating DA transmission to and reception in the torus. Our results show that only D2Rs are found in the torus though D1R labeling was present in non-auditory structures including the lateral forebrain bundle and the nucleus of the solitary tract. These results fill critical gaps in our knowledge concerning the neural basis of signal recognition and the role of DA in this process.

Acoustic Communication in Anurans

Frogs produce a number of different sounds dependent upon behavioral context. Of these, the mating call, produced only by males, has received the most attention. Mating calls serve to attract females for mating as well as identifying neighboring males for the maintenance of territorial boundaries (Capranica 1965; Rand 1988). Amidst an environment of multiple species' calls, individuals must be able to discriminate between them and respond accordingly.

The bullfrog must be able to recognize the spectral and temporal features of mating calls such as carrier frequency composition, spectral energy distribution and temporal pattern (Fig. 1). Identification and discrimination of these features can elucidate information about the emitting organism depending on the purpose of the call. Much of what we know about the acoustic cues used to discriminate between different species calls comes from early work on bullfrogs. Dr. Capranica pioneered this field with his seminal dissertation on evoked vocal responses and created a new standard for bioacoustic research. Prior to his work, field studies had shown that most species emit distinct vocalizations for species-specific communication. However, there was little neurophysiological research to support the observations made by behaviorists. Recreating the sounds found in a natural environment, Dr. Capranica found that from a chorus of 34 different species the male bullfrog only responded to bullfrog calls indicating a highly selective response that was stable and repeatable. From there he sought to distinguish the spectral patterns important for male bullfrog recognition using synthetic stimuli. Positing that not all features of the advertisement call were necessary for species recognition, he isolated specific acoustic features of the bullfrog advertisement call and presented them to male bullfrogs to determine which were most critical. A mall bullfrog could emit a highly selective vocal response known as an evoked-calling response to other bullfrog advertisement calls. By presenting a male bullfrog with isolated spectral components of the advertisement call, Capranica determined the spectral features and temporal periodicity requirements of a synthetic call.

A salient feature of a natural or synthetic sound required to evoke a call response is the presentation of concomitant energy in both the low-frequency (typically below 500Hz) and high-frequency (700 to 2000Hz, typically around 1500Hz) ranges. The spectral amplitudes could vary from -40 decibels (dB) to +10 dB relative to each other, but sufficient energy must have been present in both regions. However, an evoked response could be suppressed if the amplitude of the middle frequency range was higher than that of the high frequency component. Additionally, the optimum waveform periodicity of the call was 100 waves/second. In the bullfrog, the stimulus call, natural or synthetic, must have contained both high- and low-frequency spectral components and a temporal repetition rate of 100 waves/second in order to evoke a call response from a male bullfrog (Capranica 1965).

Anuran Auditory System

The bullfrog brain is an elongate structure, linearly organized (Fig. 3). The torus is situated beneath the tectum and underlying ventricle within the mesencephalon. A large difference between mammals and anurans is the breakdown of the cortical makeup of the forebrain. Sensory regions in higher vertebrates are divided into specialized cortices in the cerebrum. The anuran telencephalon is void of any pallial telencephalic analogs to mammalian and avian brains. Ascending, descending, audioendrocrine and audiomotor pathways are vastly spread across the anuran brain, which is indicative of the importance of social acoustic communication to all neural functions (Fig. 2).

The main route of sound conduction in anurans is through the external tympanic membrane, to the ossicles of the middle ear then to the inner ear where sound is converted into neural impulses and sent along the 8th nerve. The amphibian inner otic labyrinth contains two unique sensory organs at its posterior end, the basilar papilla and amphibian papilla, which are responsible for receiving sound waves. The basilar papilla, which is the homolog of the mammalian cochlea, conducts high frequency (between 1000 and 2000 Hz) information to the 8th nerve, while fibers tuned to lower and mid range frequencies (below 1000 Hz) innervate the amphibian papilla (Feng et al. 1975). The 8th nerve conducts neural information to the primary auditory nucleus, the dorsal medullary nucleus (DMN) and bilaterally from there to the superior olivary nucleus (SON) (Will et al. 1985). The DMN also provides contralateral collaterals to the nucleus of the lateral lemniscus (NLL) (Feng 1986), and reciprocal commissural connections to the contralateral DMN (Grofová and Corvaja 1972; Will et al. 1985; Feng 1986). Ipsilateral projections from the DMN to the toral nuclei are discussed below. The SON projects to the midbrain auditory center, the torus (see below), with collaterals also terminating in the NLL (Feng 1986). A small group of fibers has been shown to project to the caudal thalamus (Rubinson and Skiles 1975; Feng et al. 1986). From there the torus relays the information to thalamic and forebrain nuclei, where convergence of sensory, audiomotor and audioendocrine pathways take place (Fig. 2).

The torus is an obligate synapse for all descending and ascending auditory pathways; thus, it is an essential integration point in the auditory system. Compared to higher vertebrates, the organization of the bullfrog midbrain is relatively simple, making it a good model to study neural mechanisms mediating sound recognition. It is comprised of three nuclei including the laminar nucleus (Tl), principle nucleus (Tp) and magnocelluar nucleus (Tm) (Potter 1965). The Tp receives most of its ascending input from the contralateral DMN, ipsilateral SON and NLL (Feng and Lin 1991; Wilczynski 1981). The Tm and Tl receive weaker projections from these structures.

All of the forebrain projections of the toral nuclei described below are to ipsilteral regions, and they also project to their contralateral counterparts (Feng and Lin 1991; Matesz and Kulik 1996). Projections from the toral nuclei include the following: Tp and Tl project to both auditory and non-auditory regions of the brain (Fig. 2A). The Tp projects the ventromedial thalamic nucleus (VM), the central thalamic nucleus (C) and the suprachiasmatic nucleus (SC). The Tl sends afferents to anterior, central and posterior dorsal thalamic nuclei, the VM and two telencephalon structures, the striatum (Str) and the septum (Sep) (Figure 2A).

Auditory information then flows from the Sep and Str bilaterally to the posterior thalamic nucleus (P), anterior thalamic nucleus (A), the C, and ipsilaterally to the VM (Endepols et al. 2005). The P projects ipsilaterally to all toral nuclei and contralaterally to the Tl. The C and VM connect ipsilaterally to the Tl and Tm (Fig. 2B) (Feng and Lin 1991). Audiomotor signals come from the Tl to lower brainstem motor centers and to the pretrigeminal nucleus (Prv) where the pathways terminate resulting in phonotaxis (Fig 2D). The torus plays a central role in audiomotor integration as well. Ascending information from all nuclei converge on the secondary isthmal nucleus (Sl), which is relayed to the hypothalamus (Hyp) and preoptic area (PoA) (Fig 2C).

Dopamine and Receptors

DA is a neurotransmitter found throughout the brain and whose impact on various brain functions differs accordingly (Phillips et al. 2008). DA has primarily been associated with a role in reward-based learning in the limbic system and with its role Parkinson's disease. This neurologic disorder is characterized by dysfunctional dopamine signaling associated with auditory hallucinations and problems processing speech including approximating the time intervals of aural speech signals (Gräber et al. 2002 and Kantrowitz et al. 2015). This indicates, in part, that changes in normal DA levels alters the neural representation of sound and thus, DA is required for proper processing of auditory information.

DA is classified as a slow-acting neurotransmitter due to its ability to increase intracellular levels of cAMP, which was determined to be through binding to G-protein coupled receptors (GCPRs). Because there is no reuptake of dopamine by the presynaptic cell, it can diffuse to surrounding neurons and thus alter the activity and response properties of a population of cells (Binder et al. 2009). Neuromodulators are not dichotomized into standard excitatory or inhibitory categories because they alter the neuron's response depending on its functional state (Girault and Greengard 2004). Binding of DA to GCPRs initiates cascades of biochemical reactions leading to the increase in intracellular messengers and kinases such as cAMP (Kebabian and Greengard 1971). This is in opposition to classical, fast acting synaptic neurotransmitters that cause

intracellular changes of ion concentrations by binding to ligand-gated ion channels (Greengard 2001).

DA is classified as a catecholamine neurotransmitter along with adrenaline and noradrenaline. In the synthesis pathway, tyrosine hydroxylase (TH) is the rate-limiting enzyme for the conversion from L-DOPA to DA and thus, is a common way to identify catecholaminergic cells. Additional enzymes and methods can be used to distinguish dopaminergic, noradrenergic and adrenergic neurons from each other (Gonzalez and Smeets 1994).

In order to elicit a cellular response, a neurotransmitter must bind to receptors on its target cells. DA receptors can be classified into 2 groups: D1-like receptors and D2-like receptors, both of which are GCPRs making them metabotropic (Beaulieu and Gainetdinov 2011). Subtypes D1/1A, D1C, D1D and D1B/5 fall into the D1-like family; and D2, D3 and D4 subtypes comprise the D2-like group. The structure and function of these subtypes are evolutionarily conserved from invertebrates to vertebrates (Callier et al. 2003, Mustard et al. 2005 Yamamoto and Vernier 2011). D1-like receptors are located post-synaptically and inhibit adenylate cyclase via G_s proteins, which ultimately can lead to inhibitory or excitatory actions (Beaulieu and Gainetdinov, 2011; Kebabian and Calne, 1979). D2-like receptors are located both pre- and post-synaptically and are couple to G_i proteins that inhibit adenylate cyclase, and mediate inhibitory neurotransmission (Usiello et al. 2000).

DA input to the midbrain in higher-order vertebrate models is not well understood. Recently, Nevue et al. 2016 demonstrated that the only dopaminergic input to the inferior colliculus in the murine model is from the subparafascicular thalamic nucleus. Furthermore, exogenously applied DA into the IC of mice alters neuronal response properties in a heterogeneous way (Gittelman et al. 2013). A similar study conducted in bullfrogs, showed that DA applied to neurons in the torus can lead to a call-specific change in neuronal firing rate in a heterogeneous manner in response to conspecific and sympatric mating calls 75% of the time (Hall et al. 2016). With this finding in mind, the source(s) of dopaminergic input to the torus in the bullfrog is an important step towards understanding the cellular action of DA in auditory processing.

2. MATERIALS AND METHODS

Subjects

Adult, male bullfrogs, *R. catesbeiana*, were used for these experiments. Frogs (5 – 6 inches snout-vent length), supplied by Niles Biological Inc., were housed in tanks with wet and dry areas under a 16 hour:8 hour light:dark cycle. They were fed adult crickets every 7 days, flushed with fresh water daily, and treated with 2 mg of tetracycline once per day to prevent infection from *Aeromonas hydrophila*. All procedures were approved by the University of Tennessee Animal Care and Use Committee and are in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Brain Extraction & Sectioning

Five adult, male bullfrogs were used. They were anesthetized by immersion in 0.1% MS-222 buffered with 1.75% sodium bicarbonate. Complete general anesthesia was determined by lack of induced eye blink reflex and lack of leg response with toe-pinch test. Animals were perfused transcardially with 100ml of 0.01M phosphate buffered saline (PBS; pH7.4) followed immediately by 100mL 4% paraformaldehyde (PFA; App. B & C). Brains were subsequently removed, put in a scintillation vial and stored horizontally in 4%PFA at 4^0 C for 24 hours. They were taken to NeuroScience Associates, Inc. (App. D) for a proprietary protocol termed MultiBrain Processing (NeuroScience Associates 2017a) and embedded in a block of proprietary composition in the arrangement depicted in Figure 4. Two brains were oriented so that slicing yielded coronal slices and three brains were oriented so that slicing yielded sagittal slices. As one unit, they were serially sectioned at 35 mm and every 12^{th} section was collected and collated into proprietary cryprotectant at -20^0 C (NeuroScience Associates 2017b) to yield 12 complete sets of slices.

Immunohistochemistry

All immunolabeling procedures were performed on free-floating sections with agitation and at 22^0 C. Initially, the sections were rinsed with 0.01M PBS (pH 7.4) then post-fixed in 4% PFA for 55 min. Sections were then rinsed in 50% formic acid to ensure that the proteins of interest were unfolded and exposed to the surface of the sections followed by incubation in 0.5mM ascorbic acid in 0.1M ethanolamine for 10 minutes to increase the permeability of the tissues to the antibodies. Then the sections were blocked in tris-bufferred saline with 0.5% Triton X-100 (TBST) at pH 7.4 before being incubated over night in one of the

following primary antibodies: Rabbit anti-Tyrosine Hydroxylase (Millipore AB152), Rabbit anti-Dopamine D2 Receptor (Millipore AB5084P), and Rabbit anti-DRD1 (ThermoFisher 720358). Antibodies for anuran brain tissue were selected based on the processes outlined in Appendix C. To optimize antibody concentration, sections were processed at dilutions of 1:27000, 1:9000, and 1:3000. The optimum concentration was interpolated to be 1:5000 (Dr. Alexander Osmand, personal communication). Staining that was too intense and obscured details was discounted, while also excluding dilutions that were to faint so that neuronal details could not be visualized.

After 15 hours, sections were cleared of primary antibody with four 15-minute washes in 0.01M TBST (pH 7.4), then incubated in Biotinylated Goat anti-Rabbit IgG (Vectastain Elite ABC kit PK-6101) secondary antibody for 2 hours. To amplify the signal, sections were treated with a complex of Avidin DH and biotinylated enzyme for 1 hour. After serial washes in TBST (pH 7.4) the sections were treated with 0.05M Tris/0.05M imidazole (TI) buffer at pH 7.3 to optimize the reactivity of glucose oxidase. A subsequent wash of TI buffer with added 0.6% nickel sulfate heptahydrate termed, “substrate buffer”, was performed before adding the reactive solution. The reactive solution of nickel-DAB-glucose was made in substrate buffer (App. B) and the conversion of the DAB to violet-black deposits occurred over the following 30 minutes. Once the predetermined amount of intensity of the deposits was reached, as seen by viewing the sections under a dissecting microscope, they were transferred to deionized water to halt the reaction and rinsed with additional water washes. Sections were then processed using a series of ethanol dehydration steps followed by clearing of the alcohol with xylene substitute and immediately mounted on 0.5% gelatin-subbed slides using mounting media (App. B) and air dried for 24 to 48 hours. Once completely dry, the slides were coverslipped using xylene substitute mountant and air dried for 24 to 28 hours before imaging.

Immunolabeling was visualized with light microscopy using an Olympus BH201 stereomicroscope equipped with an Olympus America digital camera, and digitally acquired using the MagnaFire SP imaging software (App. B & C).

3. RESULTS

Dopamine Receptors

D2R-labeled puncta were present throughout the entire magnocelluar nucleus and clearly outlined cell somata so that they were evident against the background (Fig. 7B). The laminar nucleus contained distinct puncta, however based on visual inspection, they were not as dense as in the magnocellular nucleus and were found on neurites rather than the cell body (Fig. 7A). The principle nucleus contained the least intense staining and a sparser distribution of puncta (Fig. 7C).

There was no D1R labeling in the torus. D1R staining was limited to 2 regions in the brain, neither of which are known to be involved in auditory processing. A small population was found in the lateral forebrain bundle, and immunopositive cells were found along the entire length of the nucleus of the solitary tract.

Tyrosine Hydroxylase

TH immunolabeling of somata and neurites within the bullfrog brain tissue was present in various regions throughout the brain. Working rostral to caudal, the first region of staining was seen in a dense population of cell bodies in the glomerular layer of the olfactory bulb, which lies adjacent to a border of cell bodies on the medial edge of the anterior accessory olfactory bulb. There were a few labeled cell bodies in the internal granular layer lateral to the ventricle, and a group of labeled cells in the mitral cell layer located in the ventromedial region of the olfactory bulb. At the caudal end of the olfactory bulb there was a small number of labeled cells in the caudal extension of the glomerular layer. Representative TH-labeled cells within the olfactory bulb and accessory olfactory bulbs were shown in Figure 5A and B, respectively. The telencephalon proper was devoid of any TH-positive cell bodies.

The preoptic area was the first structure to appear in the diencephalon that had somata positive for TH. It emerged around the third ventricle and contained visually dense staining of cell bodies lining almost the entire border of the third ventricle with the exception of its ventral aspect. These cells were liquor-contacting cells with club-shaped apical intraventricular nerve processes (Fig 5D). Superior to the preoptic area was the medial amygdala, which contained a laterally-running population of TH-positive cells that lie on the ventral border of the intercerebellar space. These cells had very intense staining of the somata and axons (Fig 5C).

At the rostral chiasmatic level of the diencephalon three structures contained TH-positive cells bodies including the magnocellular preoptic nucleus, the suprachiasmatic nucleus and the optic chiasm-adjacent areas. Moving caudally the magnocellular preoptic nucleus appeared ventral to the preoptic area, gradually extending dorsally as it replaced the preoptic area. Based on visual observation, the suprachiasmatic nucleus, ventral to the magnocellular preoptic nucleus, appeared to contain relatively similar numbers of TH-positive cells as the magnocellular preoptic nucleus. On the ventral border of the diencephalon was the optic chiasm. Regions adjacent to the dorsal and lateral borders of the optic chiasm contained densely stained and packed cell bodies (Fig 5H). Spanning the dorsal-ventral length of the dorsal hypothalamus was a region of TH-positive cells bodies with lateral axonal projections (Fig 5K).

The thalamic structures comprised a majority of the dorsal part of the mid to caudal diencephalon. Based upon cytoarchitectural differences, there are two major regions, the dorsal thalamus and the ventral thalamus. The ventral thalamus contained TH-stained cells with apical extensions lining the third ventricle (5D). A majority of the ventral thalamus was occupied by the ventromedial thalamus, which spanned the length of the thalamic structures. The ventromedial thalamus contained a number of immunoreactive somata with long, lateral projections (Fig. 5I). Of the dorsal thalamic nuclei only the central and posterior nuclei contained TH-positive cell bodies in their most dorsal aspect (Fig 5J and L).

There were no TH-labeled cells in any of the three subdivisions of the torus. However, TH-labeled fibers and axons were seen throughout the subnuclei. Synaptic boutons were present along the axon tracts within all 3 subnuclei (Fig. 6)

At the caudal end of the mesencephalon, the caudal border of the anteroventral tegmental nucleus contained a small group of TH-positive cell bodies. In the brainstem, a group of immunoreactive cell bodies could be seen lateral to the isthmic nucleus. The most caudal location that immunopositive cell bodies were found was on the ventromedial border of the solitary tract. Representative cells from each TH-positive region were seen in the photomicrographs of Figure 5M, N, and O, respectively.

4. DISCUSSION

Dopaminergic System in the Bullfrog and Comparison to Other Lower Vertebrates

The main goals of this study were to provide detailed information on the possible sources of dopaminergic input to the torus semicircularis, and dopamine receptor identity and distribution within the torus. The male bullfrog, *R. catesbeiana*, has been used as a model organism for acoustic behavioral and neurophysiological research with evoked-calling responses and with neuronal response properties to acoustic stimulation post DA administration (Capranica 1965 and Hall et al. 2016). These results not only support the role of dopamingeric involvement in auditory circuits in bullfrogs and amphibians, but also provide sites for DA regulation and the distribution of toral DA receptors. While TH labeling of somata was noted in several regions of the brain, only seven have been shown to project to the torus including the suprachiasmatic nucleus, dorsal hypothalamus, ventromedial thalamic nucleus, posterior thalamic nucleus, central thalamic nucleus, anteroventral tegmental nucleus, and the ventromedial border of the solitary tract. Because TH synthesizes DA, which can proceed to form norepinephrine, the TH-positive neurons may be producing norepinephrine in addition to or instead of DA. These results are compared to other immunohistochemical dopamine studies in amphibians and reptiles.

The most rostral structure within the diencephalon that has been shown to project to the torus in bullfrogs and male northern leopard frogs (Wilczynski 1991; Feng and Lin 1991) is the suprachiasmatic nucleus. Specifically, Wilczynski's study traced the projections to both the laminar and magnocellular nuclei of the torus. TH immunoreactivity in the cell soma was also reported for the túngara frog, *Xenopus laevis*, the African clawed frog, and *Triturus cristatus carnifex*, the crested newt (Gonzalez and Smeets 1994; Franzoni et al. 1986). The African clawed frog, the marsh frog, and the Iberian ribbed newt possess cell bodies positive against TH antiserum and DA antiserum, indicating these catecholaminergic cells produced DA (Gonzalez et al. 1994; Gonzalez and Smeets 1991). Thus, the suprachiasmatic nucleus is the most rostral lying structure in the amphibian brain that could be providing dopaminergic input to the laminar and magnocellular nuclei of the torus.

Another structure within the diencephalon known to project to the torus in the northern leopard frog is the dorsal hypothalamus. Feng and Lin (1991) conducted tract tracing study in the northern leopard frog where they performed single unit recordings within specific nuclei of the torus followed by iontophoretically injected HRP. By applying a hyperpolarizing current during electrode insertion and recording, they were able to prevent HRP from diffusing

into regions other than the targeted site. These injections revealed a projection from the dorsal hypothalamus to the laminar and magnocellular toral nuclei. A similar study conducted in the bullfrog used horseradish peroxidase injections to map afferent connections to the torus (Wilczynski (1981). Occasionally cells within the ventral hypothalamus were stained, indicating a toral connection. The author noted, the cell staining occurred after large injections that invariably leached from the torus into the tectum. The cells in the bullfrog, therefore may have been projecting from the ventral hypothalamus to the optic tectum. However, an early study in the bullfrog using antisera to TH and dopamine beta-hydroxylase (DBH) showed neurons reactive for TH, but negative for DBH within the caudal region of the hypothalamus in the dorsal infundibular region (Yoshida et al. 1983). DBH is the enzyme responsible for conversion of DA to norepinephrine. The absence of DBH indicated that those cells were most likely dopaminergic. Thus, the cluster of TH-positive somata found in the dorsal hypothalamus in this study could project to the torus and could be dopaminergic. The same immunohistochemistry was performed in the reptile *Anolis carolinensis* with mirrored results (Lopez et al. 1992), suggesting cross species conservation of enzyme expression. Furthermore, the brains of four additional reptile species exhibited TH immunoreactive cell bodies within various regions of the hypothalamus (Smeets et al. 1986; Smeets 1988; Smeets and Steinbusch 1989, 1990; Smeets and Jonker 1990) suggesting phylogenetic conservation of enzyme expression. However, there were no TH-positive somata in túngara frog, the African clawed frog, or the urodele *Pleurodeles waltii* (Gonzalez and Smeets 1991, Gonzalez et al. 1993, O'Connel et al. 2010).

Of the thalamic structures, the posterior and central thalamic nuclei of the dorsal thalamus, and the ventromedial nucleus of the ventral thalamus contain cell bodies positive for TH and have been shown to project to all 3 ipsilateral toral nuclei (Feng and Lin 1991). Within the dorsal thalamus the central and posterior nuclei both contained a population of TH-positive neurons at their dorsal aspects. The staining in the posterior thalamus is highly conserved across amphibians. In the marsh frog, the cells positive for TH are also positive for DA via DA antiserum (Gonzalez and Smeets 1991). The positive DA staining in another frog species, lends a supplemental source of optimism that the posterior nucleus produces DA input to the torus. Although the bullfrog is the only amphibian with TH immunoreactive cell bodies in the central thalamic nucleus, this region has been shown to project to all three nuclei of the torus (Feng and Lin 1991; Hall and Feng 1987).

The ventromedial thalamus has been shown to project the ipsilateral Tm and bilaterally to the TI (Feng and Lin 1991). Although frogs of other genera do not display dopaminergic activity in the form of TH and DA immunopositivity within the ventromedial thalamus, the marsh frog, a ranid species, did (Gonzalez and Smeets 1991). The marsh frog showed a small group of cell bodies reactive to TH antiserum. This corresponds to the results presented here. The marsh frog

did not show reactivity to dopamine antiserum. The lack of positive staining indicated the formation of catecholamines further down the synthesis pathway..

Three regions caudal to the torus contained TH-positive cell bodies including the dorsal border of the anteroventral tegmental nucleus and the ventromedial border of the solitary tract. The anteroventral tegmental nucleus is known to the project to the laminar and magnocellular nuclei of the torus. In addition to having TH-positive cell bodies in the bullfrog, the tùngara frog, African clawed frog and two species of newt contained TH reactive cell bodies as well (O'Connell et al. 2010; Franzoni et al. 1986). However, the marsh frog did not show immunoreactive cells bodies in this area (Gonzalez and Smeets 1991). There is no data for the ventromedial border of solitary tract in the tùngara frog because the study did not include these areas. The most caudal region that expressed TH-positive cell bodies in the bullfrog was the ventromedial border of the solitary tract, which has been shown to project to all three toral nuclei (Feng and Lin 1991). Both the African clawed frog and the Iberian ribbed newt contain TH-positive cells bodies in this region and DA-positive cell bodies in relatively the same amounts upon visual inspection (Gonzalez and Smeets 1991). An early study in the bullfrog, found the solitary tract to contain TH-positive cell bodies that were also DBH-positive and phenylethanolamine N-methyltransferase (PNMT)-positive indicating that these cells convert DA to noradrenaline and adrenaline (Yoshida et al. 1983). These neurons could have produced DA at some point, but when DBH is present that DA is converted to noradrenaline, therefore it is unknown whether these cells were dopaminergic.

This study was one of few that used a polyclonal antibody again TH, whereas a majority of previous studies used antisera against TH and one study used a monoclonal antibody against mouse TH. The use of antisera versus antibodies can affect binding specificity within the neural tissue. Similarly, if the antiserum is raised against a mammal as the monoclonal antibody was, then the two paralogs that encode for TH in all other vertebrates is not taken in account (Yamamoto et al. 2010). In addition, species differences could account for the variation seen in TH staining among the frogs.

Dopamine Receptors

In the higher vertebrates such as rats and humans, the inferior collciulus contains D2, but not D1 receptors (Wamsley et al. 1989; Hurd et al. 2001). Here it's shown that the bullfrog torus solely contains D2Rs. This is the first study utilizing a primary antibody against the D1 receptor to visualize it presence in the anuran torus. Dopamine and cAMP-regulated phosphoprotein-32 (DARP-32) has been used a marker for dopamine-receptive cells acting via the D1-like receptors. DA binds D1-like receptors leading to an increase in cAMP levels and subsequent activation of protein kinase A, which phosphorylates DARP-32

(Walaas and Greengard 1984). O'connell et al. 2010 showed that all three toral nuclei contained cells immunopositive for DARP-32 in the Túngara frog. Their observation is in direct opposition with what these results have shown ie. the torus does not contain D1Rs. Because only the D2R has been sequenced in bullfrogs, the western clawed frog protein sequence that was used to assess similarity to the antigen sequence may have been too dissimilar to probe for D1R. A draft genome was reported for the bullfrog in early 2017(Hammon et al. 2017). If it contains the sequences for the DA receptors, then the draft genome can be used for further exploration into the receptor-antibody compatibility.

The D2R staining in the magnocellular nucleus of the torus compared to the principle and laminar nuclei is more prominent such that the somata and cell body projections are distinguishable. It is unclear whether the D2R puncta in the laminar nucleus and magnocellular nucleus were solely on the postsynaptic cells or whether they could have been located presynaptically. This will require electron microscopy of the staining to assess. A study using *Bombina orientalis*, the fire-bellied toad, *Discoglossus pictus,* the painted frog, and the African clawed frog used a D2R antibody raised in rabbit to look at the receptor distribution in the auditory midbrain. They were able to stain soma and basal dendrites, noting the high intensity of staining and consistency among the species (Endepols et al. 2000). The consistency can now be extended to the bullfrog, based on the staining displayed in this study.

Functional Implications of Dopamine in the Auditory System

It has been previously shown that there are neurons within the torus selective for the bimodal frequency spectrum of the species' call (Fuzessery and Feng 1982). This frequency selectivity capability can recognize components of the species mating call. Frequency selectively can also be stimulated by manipulating the neurotransmitter γ-aminobutyric acid (GABA) receptor activity (Hall 1999). After iontophoretically applying the GABAa receptor blocker bicuculline methiodide to the torus, the response properties of toral nuclei were characterized. GABAergic inhibition affected frequency curves and stimulus evoked discharge rate, among other properties. While it is known that GABAergic inhibition plays a role in frequency selectivity, it is also possible that DA contributes to this process.

Recently, the response of neurons in the torus to recordings of conspecific calls and sympatric species calls, both before and during iontophoretic application of dopamine, revealed 3 response categories neurons in the torus. When presented with the conspecific bullfrog mating call plus DA application, neurons within the torus showed either enhanced or suppressed evoked activity. The call of a sympatric species, the green frog, did not evoke a change in the firing rate of bullfrog neurons in the torus. Results revealed call-dependent

effects on discharge rate (Hall et al. 2016). A similar study conducted in the mouse inferior colliculus, found that DA modulates auditory responses in a heterogeneous manner as well providing evidence for DA's functional conservation in vertebrates (Gittelman et al. 2013).

The subparafasicular nucleus located in the posterior thalamus of mice, has been shown to be the source of dopaminergic input into inferior colliculus. (Nevue 2016) The region with TH immunopositive cell bodies in the posterior thalamic nucleus is a likely anuran analog due to the analogous midbrain structures between mice and anurans. Ponnath and Farris 2014 conducted a study measuring the activity of individual neurons within the torus when stimuli, in the form of repeated blocks of an electrical stimulus, followed by an acoustic stimulus followed by silence was presented to the thalamus. Congruent with the findings from Hall et al. 2016, evoked activity would change heterogeneously per neuron, increasing or deceasing. In addition, they found that latency could be shifted due to thalamic stimulation. This information presents a solid case for the conclusion that the posterior thalamic nucleus is a logical region of DA input to the torus. At the level of the torus, all 3 nuclei contain axonal tracts and synaptic boutons that are positive for TH indicating they all receive dopaminergic input.

In *Hyla cinerea*, the green treefrog, neurons within the ventral hypothalamus show an increase in excitatory responses to conspecific mating calls and receive descending auditory input from the central thalamic nucleus (Allison 1992; Allison and Wilczynski 1991). As an integral structure in the limbic system the hypothalamus also receives input from the medial striatum and septal complex, making it a key point of convergence for limbic and audioendocrine pathways (Neary 1995). These strong thalamo-hypothalamic pathways are present in homologus structures in rats and birds as well (LeDoux et al. 1985; Cheng and Zuo 1994). As concluded here, the hypothalamus is mostly likely a source of dopaminergic input to the torus. The hypothalamus receives input from the stratum and pallium creating a link between the limbic and audioendrocrine system (Neary 1995; Endepols et al. 2005). The limbic system relays information regarding the basal drive to mate and that information prompts a response from the hypothalamus, of which could be the release of DA to the torus to modulate neuronal responses to mating calls. From the torus the information is relayed to the motors centers in the medulla and brainstem to elicit phonotaxis. The modulation that DA provides within the torus can increase or decrease the responsiveness of neurons that relay information to motor centers. For example, a bullfrog would need to move away from an area that is not producing conspecific mating calls and towards a location where its species is prevalent.

In the descending auditory pathway, the thalamus projects the principle and laminar nuclei in the torus. As with Capranica's seminal work revealing that in order to elicit an evoked calling response a natural or synthetic sound must contain both low and high frequency components, neurons in the thalamus must

also detect the presence of low and high frequency to be excited. They found that neurons in the thalamus exhibit selectivity for behaviorally relevant sounds. If these neurons then provide dopaminergic input to the torus, which has already shown to modulate neuron response properties, then a possible function of this input could be to be an additional point of modulation to select for species-specific mating calls (Fuzzery and Feng 1982). Furthermore, the spectral and temporal features relaying species identity within a mating call are processed differently and independently in the central and posterior thalamic nuclei (Hall and Feng 1987). Not only could these regions being providing another point along the processing pathway for mating call selectivity, but each nucleus is severing an independent point of different processing.

The suprachiasmatic nucleus is responsible for controlling circadian rhythms by receiving environmental cues and eliciting internal responses that coordinate physiology output. The primary environmental cue is photic information, but also includes social interactions through auditory stimulation. Auditory cues can be strong inducers of circadian modulation as seen in the house sparrow whose behavioral rhythm can be entrained by conspecific songs (Davidson and Menaker 2003). Similarly, socially monogamous songbirds will engage in extra-pair mating during the period of the dawn chorus. The time at which a male engages in singing during the pre-dawn period affects mating success, thus, appearing to be a sexually selected signal modulated by circadian light cycles (Kempenaers et al 2010; Krebs and Kacelnik 1983). In addition to vertebrates, there is a plethora of studies demonstrating the role of circadian rhythms in mating activity at the biochemical level to the behavioral level in fruit flies, moths and cockroaches. There is evidence of circadian regulation of auditory function at the level of the cochlea as well. A study conducted by Meltser and collegues demonstrated a dinural variation in the acoustic startle response and a dinural sensitivity to noise trauma, as well as the presence of a molecular clock in the cochlea in mice (Meltser et al. 2014). Because bullfrogs rely on mating calls to attract females for reproduction, it stands to reason that a possible function of the dopaminergic input from the suprachiasmatic nucleus to nathe torus is to provide circadian modulation of mating behavior.

The variety of information converging on the torus proves the torus to be an integral site for information assimilation rather than a structure for the sole purpose on transmitting auditory information the thalamus. DA input from suprachiasmatic nucleus, dorsal hypothalamus, ventromedial thalamic nucleus, posterior thalamic nucleus, central thalamic nucleus, anteroventral tegmental nucleus, and/or the ventromedial border of the solitary tract may function to modulate neuronal selectivity for behaviorally relevant sounds.

5. FUTURE DIRECTIONS

Sequencing of all the bullfrog dopamine receptor subtypes would be beneficial in identifying the sequence similarity to the antibodies used in this study. Primary antibodies can be designed to target any receptor subtypes that are not targeted by the antibodies used here, and their distribution throughout the auditory system can be mapped.

Because TH is the enzyme in the rate-determining step of DA synthesis, which is the precursor to norepinephrine and epinephrine synthesis, the regions with TH positive somata thought to project to the torus could be producing any one or combination of these neurotransmitters. These regions need to be defined as dopaminergic cells in addition to or in lieu of producing other catecholamines. A retrograde tracer needs to be iontophoretically injected into the torus of awake bullfrogs, and the brain sections stained for TH. The most common tracer used is horseradish peroxidase, however Fluoro-Gold is a non-viral option, and the pseudorabies virus with modified membrane proteins will trace in a retrograde fashion. Labeled cells whose cell body is positive for TH and terminals are TH-positive within the torus are likely the sources of DA input to the torus. Simultaneous immunostaining of these sections with dopamine beta-hydroxylase (DBH) will discriminate between dopaminergic cells and nor-/adrenergic cells that project to the torus. DBH is the enzyme responsible for converting dopamine to norepinephrine thus; DBH-negative neurons are not converting their DA to norepinephrine. This process will confirm and possibly narrow down the midbrain regions that provide dopamine to the torus. The tracing process can be essentially run in reverse with an anterograde tracer, most likely horseradish peroxidase, injected into possible dopaminergic regions. Furthermore, the brain sections can be stained with DBH and PMNT, the enzyme that converts norepinephrine into epinephrine, and the specific catecholamine produced can be determined.

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APPENDICES

APPENDIX A - FIGURES

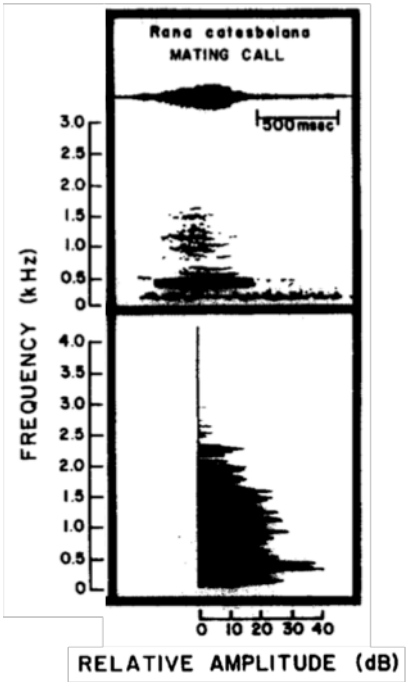

Figure 1 *R. catesbieana* mating call. Spectral and temporal characteristics of the bullfrog mating call (adapted from Feng et al. 1990).

Figure 2 Auditory pathway connections. Representation of auditory pathway connections in *R. catesbieana*. Ascending auditory pathways (A); Descending auditory pathways (B); Audioendocrine connections (C); Audiomotor connections (D) (Wilczynski and Endepols 2007).

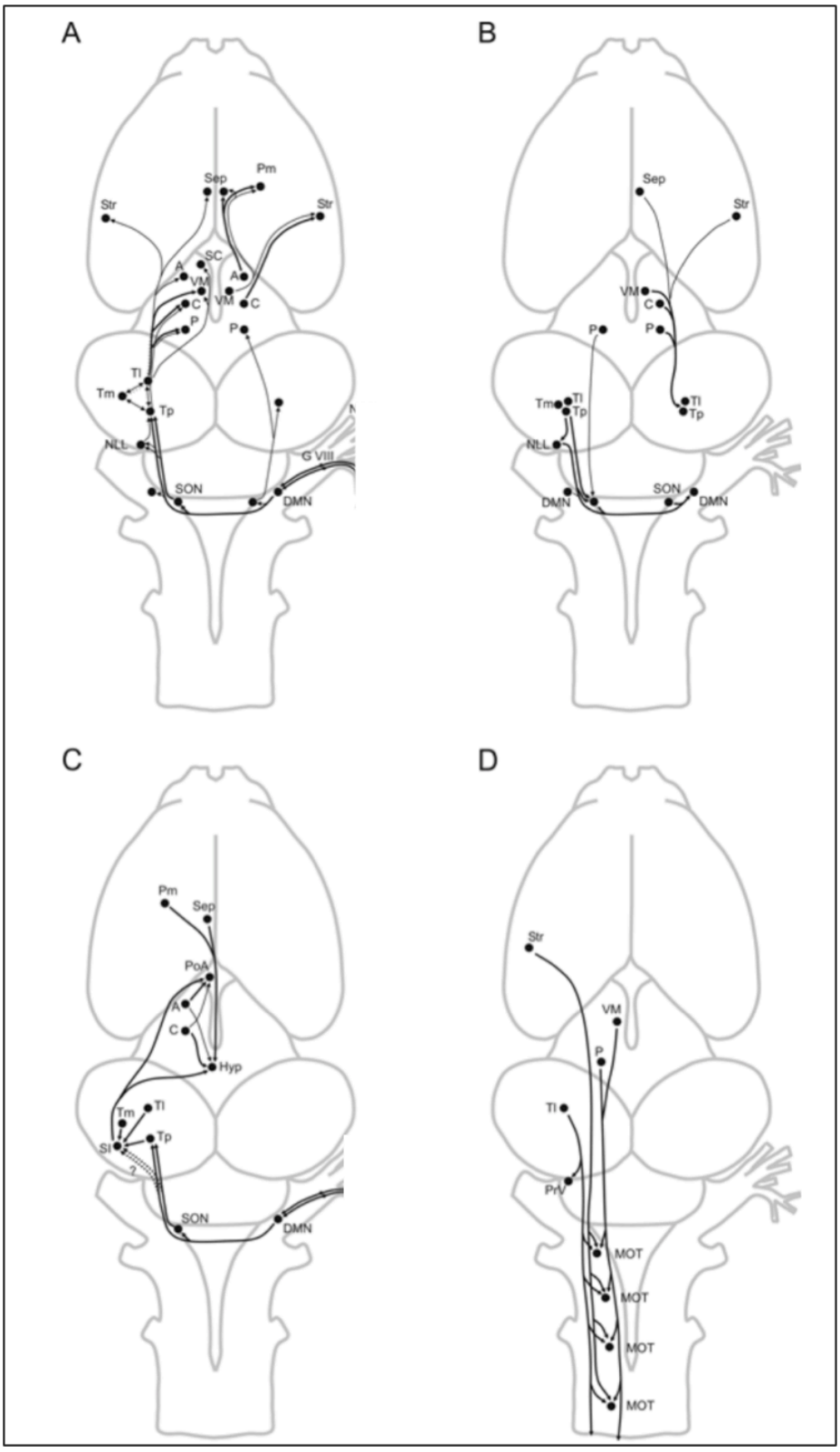

Figure 2 Continued

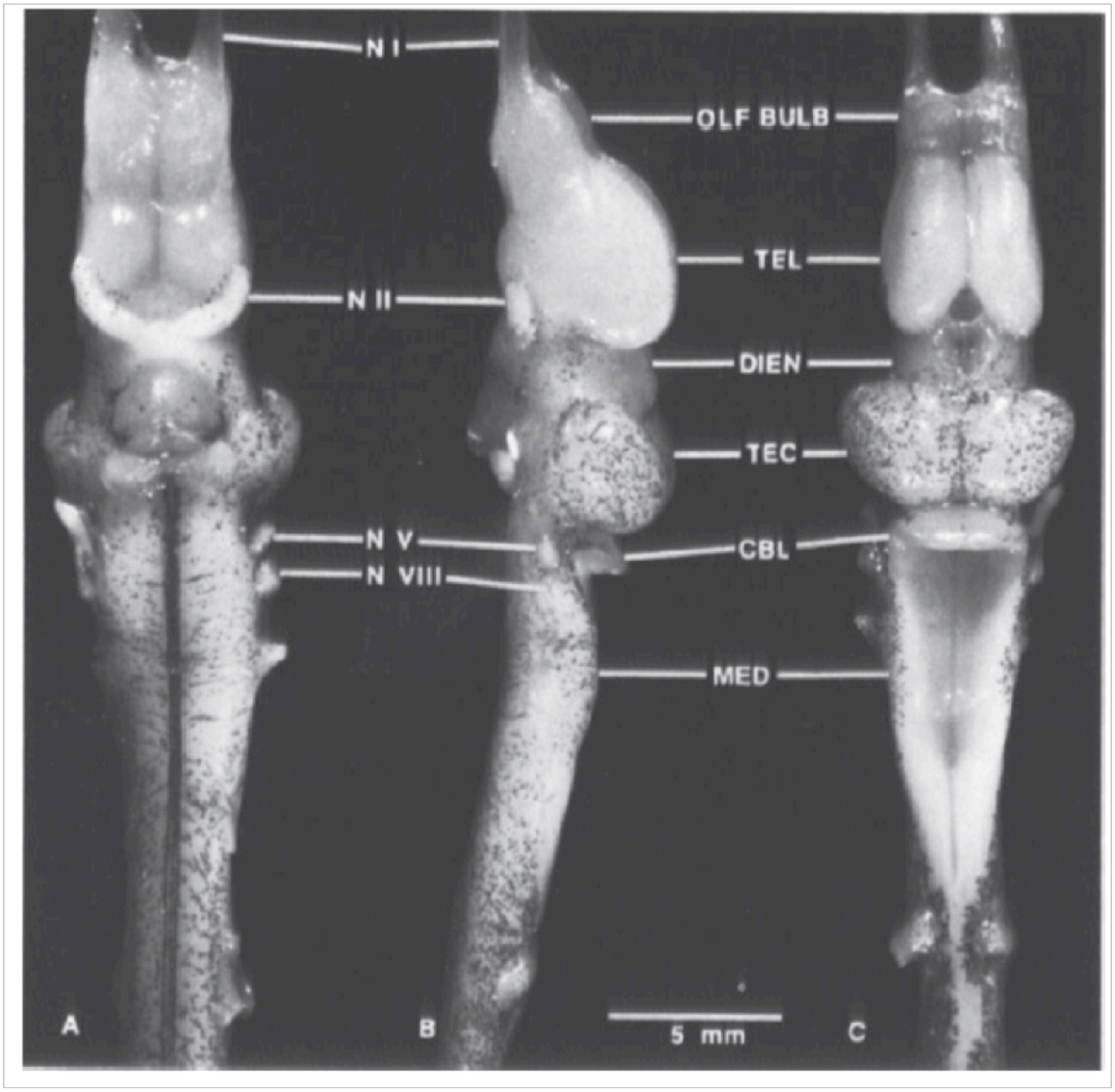

Figure 3 *R. catesbieana* brain structure. Major divisions as viewed ventrally (A), laterally (B), and dorsally (C). OLF BULB olfactory bulb; TEL telencephalon; DIEN diencephalon; TEC optic tectum; CBL cerebellum; MED medulla; N I olfactory nerve; N II optic nerve; N V trigeminal nerve; N VIII acousticovestibular nerve (adapted from Kicliter and Ebbesson 1974).

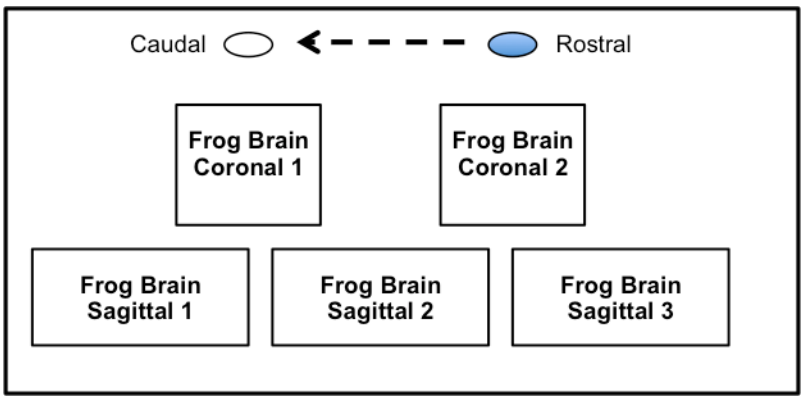

Figure 4 Brain block configuration for sectioning. Five frogs brain were embedded in gelatin block of proprietary composition. Two brains were oriented so that slicing yields coronal sections, and three brains were orientated so that slicing yields sagittal sections. Slices are 35 mm apart and collected in 12 series.

Figure 5 TH immunoreactivity outside of the torus. Photomicrographs taken from coronal sections of the brain of *R. Catesbeiana* showing typical tyrosine hydroxylase-labeled somata (arrows) in the olfactory bulb (A), accessory olfactory bulb (B), region superior to the medial amygdala (C), preoptic area (D), ventral thalamic nucleus (E), magnocellular preoptic nucleus (F), suprachiasmatic nucleus (G), optic chiasm (H), ventromedial thalamic nucleus (I) central thalamic nucleus (J), dorsal hypothalamus (K), posterior thalamic nucleus (L), anteroventral tegmental nucleus (M), region lateral to the isthmic nucleus (N), and the ventromedial boarder of the solitary tract (O). Bars = 20μm

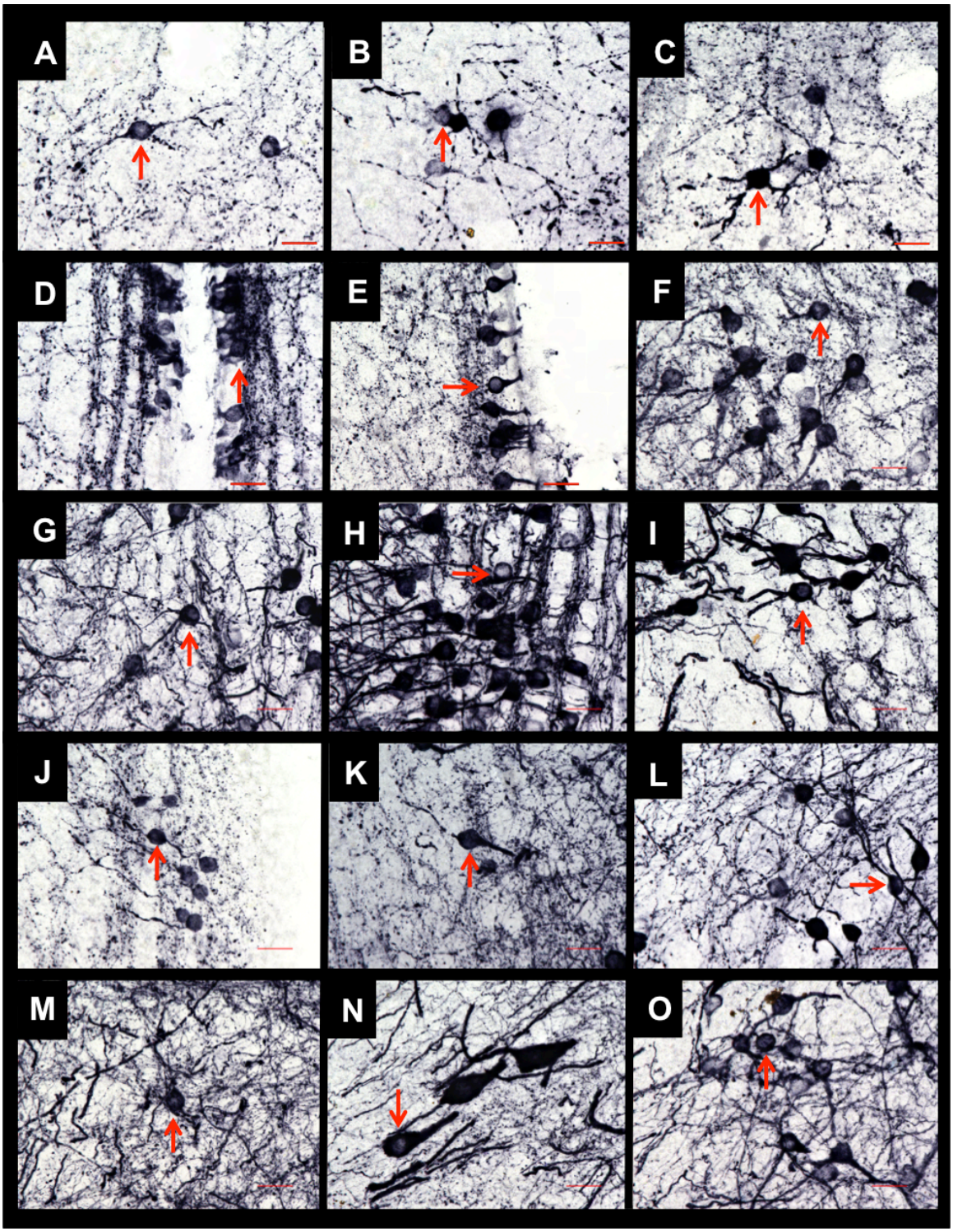

Figure 5 Continued

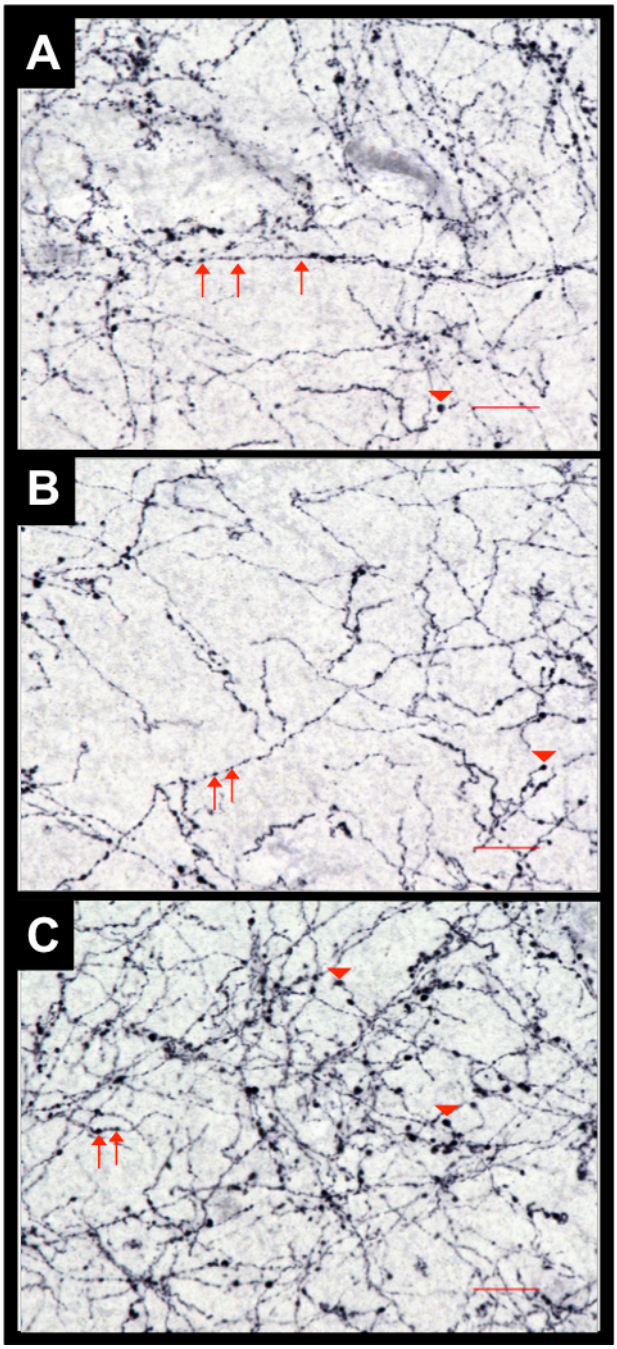

Figure 6 TH immunoreactivity in the torus. Photomicrographs of tyrosine hydroxylase immunoreactivity in the 3 subnuclei of the torus in the brain of *R. Catesbeiana* showing the laminar (A), magnocellular (B) and principle (C) nuclei. Axon tracts traverse the images. Synaptic boutons are present along axons as indicated by arrows, and axon terminals are indicated by triangles. Bars = 20μm

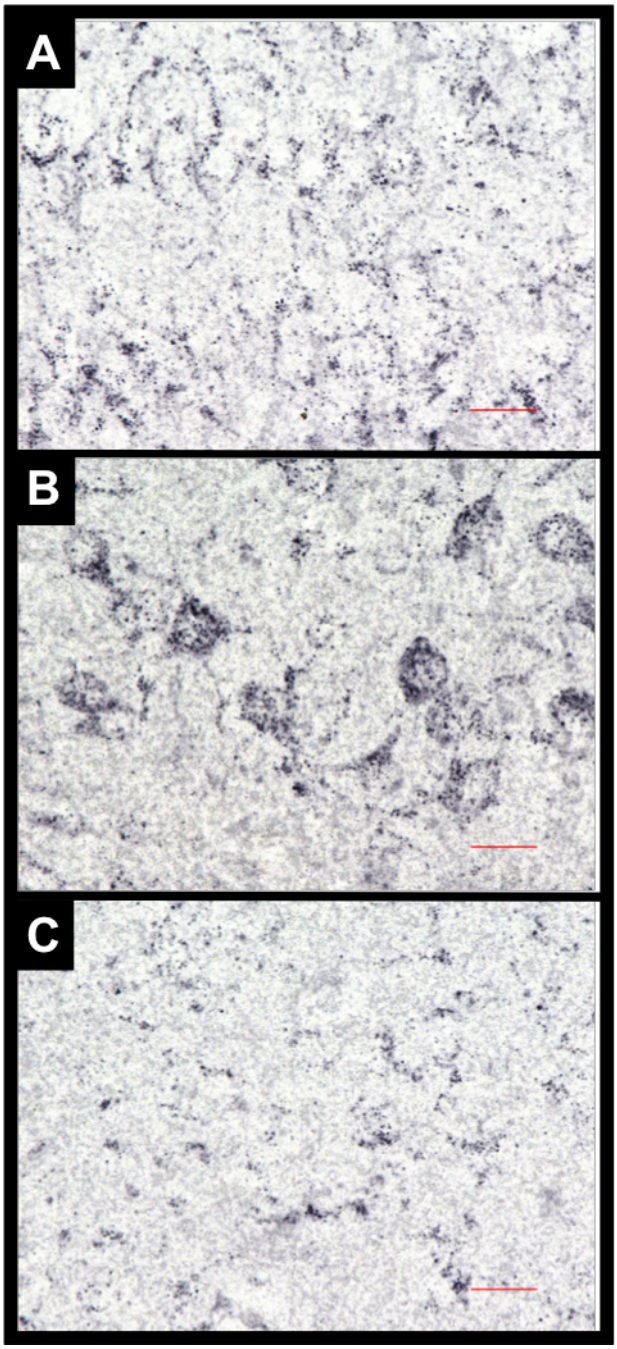

Figure 7 D2R immunoreactivity in the torus. Photomicrographs of D2R immunoreactivity in subnuclei of the torus in the brain of *R. Catesbeiana* showing the laminar (A), magnocellular (B) and principle (C) nuclei. Receptor puncta seen in various intensities and size in each nuceli. Bars = 20µm

APPENDIX B – SOLUTION RECIPES

B.1: 4% paraformaldehyde (PFA)

Make 1mL of 4% PFA in 0.1M PBS

- In a 1.5 or 2L flask - 1mL MilliQ H_2O; 40g PFA; 11.57g sodium phosphate dibasic (mw = 142g)
- Place on stir plate w/heat under hood set to '6' until ~60^0 & dissolved completely (sol will be clear)
- Turn off heat & cont. stirring until cooled (sometimes useful to switch to another stir plate)
- Add 2.6g sodium phosphate monobasic (mw 137.99) w/stirring [dissolves at room temp, but a little heat helps]
- pH & titrate; stir until dissolved
- pH & filter - store at 4^0 [must be really cold for perfusions]

B.2: Immunohistochemistry Solutions

B.2.1 Phosphate Buffer Saline with Triton (PBSTx)

1X PBS with 0.5% Triton X-100 in MilliQ H_2O

B.2.2 TI Buffer

0.05M Tris/0.05M imidazole in MilliQ H_2O
pH adjusted to ca. 7.3 with acetic acid

B.2.3 Substrate Buffer

0.05M imidazole with 0.6% Nickel sulfate heptahydrate in MilliQ H_2O
pH adjusted to ca. 7.3 with acetic acid.

B.2.4 Mounting Media

Composition: 20% ethanol, 0.1M ammonium acetate, 0.1M ammonium bicarbonate, 0.0025% pigskin gelatin in MilliQ H_2O
Stir & syringe filter

APPENDIX C – DETAILED PROTOCOLS

C.1: Antibody Selection

Choosing an antibody for use on bullfrog brain tissue took into consideration the source and sequence of the antigen that the antibody was raised against. Primary antibodies raised against bullfrog protein sequences do not exist; therefore, the animal with the closest sequence identity to the bullfrog protein was chosen. In order to do this, the sequence of the bullfrog proteins of interest needed to be determined. Only the D2R sequence has been resolved in the bullfrog, therefore the D1R and TH sequences from close relatives, found using the UniProtKB database, of the bullfrog was used for comparison.

The NCBI BLAST program was used to run multiple sequence alignments. The closest relative of the bullfrog for which the D1R sequence was known and had the highest sequence identity to the complete protein sequence where the antigen was derived was the African clawed frog, *Xenopus laevis* (UniProt P42289; Sugamori et al. 1994). Performing a multiple sequence alignment on common animals used for primary antibody production yielded an 80% identity of the African clawed frog to the human sequence, *Homo sapiens* (UniProt P21728; Dearry et al. 1990). Similarly, the closest relative of the bullfrog for which the TH sequence was known and has the highest sequence identity to the complete protein sequence where the antigen was derived was the Western clawed frog, *Xenopus tropicalis* (UniProt F6UM15; Hellsten et al. 2010). Performing a multiple sequence alignment on common animals used for primary antibody production yielded a 73% identity of the Western clawed frog to the rat, *Rattus norvegicus* (UniProt P04177; Grima et al. 1985).

In addition, the optimum primary antibodies to use were polyclonal from multiple B cell lines, which recognized multiple epitopes of the antigen of interest. Other factors taken into consideration were the purification method, the indicated application for antibody use, and the host. Affinity purification also known as chromatography purification produced the purest antibody as it was resolved by quality of its specific binding properties. The antibody data sheet must indicate that it has been proven to work in immunohistochemistry applications, and if specified, that it worked for the fixation and/or embedding media used on the tissue.

Finally, the host species that the antibodies were raised in affected the secondary antibody that can be used in conjunction with it. All of the antibodies used this work were polyclonal, affinity purified, proven to work for immunohistochemistry, and raised in rabbit. The anti-TH, Millipore AB152, primary antibody was previously characterized by O'Connell et al.

2010. Because all of the primary antibodies are raised in rabbit, the secondary antibody was raised in goat to elicit production of a goat antibody to the rabbit protein.

C.2: Transcardial Perfusion

C.2.1 Anesthetize

- 0.1% MS-222/1.75% $NaHCO_3$ in MilliQ H_2O
- Assess responsiveness to toe-pinch and eye-blink reflex

C.2.2 Bullfrog Prep

- Rinse frog off with H_2O
- Position on its dorsal side on an elevated tray with drainage, and affix with pins
- Feel for the rib cage and chest plate – cut the skin laterally across from edge of rib cage to edge of rib cage, up along the inside of the arms and across under the neck – remove the skin section
- Cut the same pattern through the musculature and connective tissue while removing the bone in this path
    - Cut the connective tissue bands holding the tissue flap to the inside
- Remove any lobes of liver that are obscuring the heart
- Use forceps to hold slightly up the opaque pericardium and the small pointed scissors to remove
- Gently lift the heart and cut the dorsal connective bands to gain access to the atria
- If the fatty layer on the ventral side of the heart is obscuring the atria, gently cut some away with the small pointed scissors
    - The ventral surface of the heart now exposes the bulbus cordis leading to the truncus arteriosus

C.2.3 Perfusion

- Using the hemostats (unclamped) to keep the heart in place, insert needle (manually or via microdrive) at a 30^O angle, 1cm into the superior aspect of the

    - Careful not to go through conus arteriosus and into the atria
    - Clamp the internal portion in place with hemostats & turn pump on
- Immediately nick the right atrium laterally with small pointed scissors
    - Blood will pump out of the atrium
- Pump 100mL of 1x PBS at 15mL/min
    - The effluent fluid from the atrium will run clear and the liver will lighten in color as the blood is replaced by buffer
- Pinch the tubing 6 inches above the end in the 1x PBS beaker, transfer to the 4%PFA beaker and release
    - This prevents air bubbles from entering the tubing and, thus, the tissue
- Pump 100mL of 4%PFA at 15mL/min
    - The extremities will start to move & push – in a non-twitch-like manner
    - Then they will stiffen, as will the internal organs

C.2.4 Brain Extraction

- Remove the lower jaw with large blunt scissors
    - Cut far back to get through the bones
- Decapitate with large blunt scissors (straight across where the lower jaw hinged on)
    - See the brain stem in the center of the cut edge
- Scrape off soft tissue on roof of mouth the expose the hard plate – this will serve as a guide for extraction
- Using large blunt scissors cut outside of, but along, the diamond-shaped opaque bone
    - Diamond-shaped w/o pointed ends and a straight section (the sphenethmoid bone) projecting rostrally
- Scrape off the skin & soft tissue off, using blunt large and medium scissors on an angle and sheering the tissue off
- On the ventral surface use medium blunt scissors to cut lateral of the midline going rostrally from the hole where the brain stem is exposed
- Use forceps to lift the center bone piece created, out of the skull to expose the brain

If the perfusion was poor or questionable, stop here and proceed to Post Removal

- Use forceps, rongeurs and small/medium scissors to remove the remaining ventral and lateral portions of skull
- Use small pointed scissors to sever the cranial nerves and extensions of the olfactory bulb
- Gently remove brain from the remaining skull

C.2.5 Post Removal

- Placement –
    - Depending on the quality of the perfusion, the brain(s) can be covered with 4%PFA in a beaker or
    - Placed in a horizontal scintillation vial and covered with 4%PFA

- Post Fixation
    - A good perfusion – 2 to 4 hours in 4%PFA post fixation
    - A decent perfusion – O/N in 4%PFA
    - A questionable perfusion – 24hr in 4%PFA
    - A poor perfusion – 1-2 day in 4%PFA

C.3: Thionine Nissl Staining

- Put slides in 50^0 C oven for 10min
- Washes

Solution	Duration
70% EtOH	3min
MilliQ H_2O	3min
Nissl Mix* – time depends on optimization of scavenger sections	
MilliQ H_2O	3min
Quickly assess sections under scope for stain leakage	
70% EtOH	3min
95% EtOH	3min
100% EtOH**	3min
100% EtOH**	3min
50/50 xylene substitute/100% EtOH	3min

Xylene Substitute #1	3min
Xylene Substitute #2	3min

*Nissl Mix = 400mL MilliQ water in staining dish

+ 10mL of 2M acetic acid
+ 10mL of 2M sodium acid
+ 2.5mL of 1% Thionine

**Dishes must be completely free of any H_2O: rinse with di H_2O; paper towel dry; bake @ 50^oC 5min before use

C.4: Modified Osmand IHC Protocol

Modified method from Osmand et al. 2006.
All steps performed on free-floating 35 micron sections at room temperature with gentle agitation.

Primary Antibodies: Rabbit anti-Tyrosine Hydroxylase (Millipore AB152)
Rabbit anti-Dopamine D2 Receptor (Millipore AB5084P)
Rabbit anti-DRD1 (ThermoFisher 720358)
Secondary Antibody: Biotinylated Goat anti-Rabbit IgG (Vectastain Elite ABC kit PK-6101)

Day 1

1) Wash 3x5min 1XPBS
2) Rinse 55min 4% PFA
3) Wash 3x5min 1XPBS
4) Wash 5min MilliQ H_2O
5) Rinse 3x10min 50% formic acid
6) Wash 3x5min 1XPBS
7) Incubate 1hr 0.1M Ethanolamine pH 9.5
8) Add 0.5mM ascorbic acid to previous step – 10min
9) Wash 3x5min 1XTBS
10) Block 10, 30, 10min 1X TBST
11) Incubate in primary antibody overnight

Day 2

1) Wash 4x15min 1XTBST
2) Wash 2hr in secondary antibody
3) Incubate 1hr in ABC reagent (1:200000 reagent A & 1:200000 reagent B ie. 1:2.5 from manufacturer's recommendation)
   a. Make 30min before use
4) Wash 15, 15, 10min 1XTBST
5) Rinse 10min 1X TI buffer
6) Rinse 10min Substrate Buffer
7) Incubate (up to 30min) Substrate Buffer + 0.02% Diaminobenzidine (DAB), 0.2% glucose, 0.004% ammonium chloride, 2g glucose oxidase
8) Wash 2x7min MilliQ H_2O
9) Transfer to fresh MilliQ H_2O into 4^0 C

Day 3

1) Transfer sections into Mounting Media for 1 to 2hrs at room temperature
2) Paint brush-mount sections onto 0.5% Gelatin subbed slide in Mounting Media
3) Air dry at room temperature for 48hrs

Day 5

1) Process
2) Coverslip

C.5: Process and Coverslip

Process: Washes

Solution	Duration
70% EtOH	3min
MilliQ H_2O	3min
70% EtOH	3min

95% EtOH	3min
100% EtOH**	3min
100% EtOH**	3min
50/50 xylene substitute/100% EtOH	3min
Xylene Substitute #1	3min
Xylene Substitute #2	15min

**Dishes must be completely free of any H_2O: rinse with di H_2O; paper towel dry; bake @ 50^oC 5min before use

Coverslip

1) Keep slides in Xylene Substitute #2
2) Remove one slide & add Xylene Substitute Mountant
3) Lower coverslip onto slide at an angle
4) Air dry at least 24 hours

APPENDIX D – AFFILIATE COMPANY INFORMATION

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F: 865-675-2787

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VITA

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