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Anatomy and Function of the African Clawed Frog Vocal System is Altered by the Brominated Flame Retardant, PBDE-209

Lisa Rania Ganser
University of Miami, lganser@gmail.com

UNIVERSITY OF MIAMI

ANATOMY AND FUNCTION OF THE AFRICAN CLAWED FROG VOCAL SYSTEM IS ALTERED BY THE BROMINATED FLAME RETARDANT, PBDE-209

By

Lisa Rania Ganser

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Lisa Rania Ganser

Approved:

Zhongmin Lu, Ph.D. Professor of Biology	Teresa A. Scandura, Ph.D. Dean of the Graduate School
Edward J. Green, Ph.D. Professor of Psychology	Kathryn Tosney, Ph.D. Professor of Biology

William A. Hopkins, Ph.D.
Professor of Biology
Virginia Polytechnic University

GANSER, LISA R. (Ph.D., Biology)
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Vocal communication allows animals to express distress, territoriality, and most important, to attract mates. In the African Clawed frog, *Xenopus laevis*, vocal communication is unique, because not only do males advertise for mates using elaborate click vocalizations, but also females are able to advertise their reproductive readiness by eliciting a "rapping" call. Sex differences in vocal repertoire match sex differences in vocal circuitry. During development, the vocal circuitry in the male grows increasingly sensitive to circulating androgens. Androgens induce tremendous growth in the cartilage and musculature of the peripheral vocal organ, the larynx. Net addition of synapses and motor fibers soon follow providing communication from the motor nucleus in the hindbrain to the vocal organ. The laryngeal motor nucleus, n. IX-X, accumulates androgens that serve to protect n. IX-X neurons from programmed apoptosis. Females, who have low levels of circulating androgens, experience a profound net loss on n. IX-X neurons during this developmental critical period. Once the frogs reach sexual maturity males possess larger and more numerous n. IX-X neurons than females, as well as sizable sex differences in laryngeal robustness and physiology. These measurable sex differences yield vastly different vocal programs. Androgens continue to maintain a critical role in governing breeding season trophic effects and mediating call production. Because male

X. laevis are so susceptible to the effects of androgens, they may also be sensitive to the actions of endocrine disrupting chemical agents. The vocal system of *X. laevis* and its androgen sensitivity thus provide an ideal model for studying changes imposed to the anatomy and physiology of the system by the brominated flame retardant, PBDE-209, a putative anti-androgen and common pollutant. The present studies investigate how PBDE-209 affects the male vocal system when animals are exposed during the androgen-sensitive critical period of vocal system development and during adulthood when the tissues are utilizing androgens to vocalize. PBDE-209 effectively reduces male n. IX-X number and size at higher concentrations after exposure during the organizational critical period. Similar dose-dependent effects were observed in adult n. IX-X neurons. Moreover, PBDE-209 inhibited male-typical vocalization by reducing the number of calls elicited as well as the average call amplitude. These data strongly suggest that PBDE-209 has cytotoxic effects that alter n. IX-X anatomy and function, and may be mediated through pathways that include blocking the androgens necessary for proper vocal system development.

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4V = fourth ventricle

Cb = cerebellum

cm = centimeter

DHT = dihydrotestosterone

DMSO = dimethylsulfoxide

DTAM = dorsal tegmental area of the medulla

EDC = endocrine disrupting chemical

EMG = electromyogram

FETAX = frog embryo teratogenic assay for *Xenopus*

GN = glottal nucleus

GVE = general visceral efferents

HCG = human chorionic gonadotropin hormone

HDPE = high density polyethylene

Hz = Hertz

IU = injection units

M = medulla

mm = millimeters

MS-222 = tricaine methanesulfonate

n. IX-X = motor nucleus of the glossopharyngeal-vagal nerve

N. IX-X = glossopharyngeal-vagal nerve

N. V = trigeminal nerve

N. VIII = vestibulocochlear nerve

PBDE = polybrominated flame retardants, polybrominated diphenyl ether

PCB = polychlorinated biphenyl

PFA = paraformaldehyde

Pit = pituitary gland

PM0 = post metamorphic month 0

PM6 = post metamorphic month 6

POA = preoptic area

ppb = parts per billion

ppm = parts per million

PTFE = polytetrafluoroethylene

PVC = polyvinylchlorine

RF = reticular formation

ST = solitary tract

SVL = snout vent length

T = thalamus

Tel = telencephalon

TH = thyroid hormone

µg = microgram

µm = micrometer

µV = microvolts

CHAPTER 1
INTRODUCTION

Animals communicate reproductive capabilities and reproductive readiness with visual, olfactory, and auditory cues. These signals ensure the animal's reproductive success, and ultimately species survival. Without appropriate cues, an animal may not be able to protect itself from predation or secure its survival through successful mating events. Many communicatory systems are organized early in the developmental process. The organization phase is orchestrated by the actions of a precise mixture of hormones that promote the growth and the interaction of the organ systems involved in sexual communication. By the time these systems are organized, measurable sex differences are apparent not only in peripheral and secondary sex characters, but also in central neural circuits and nuclei.

For frogs, reproductive readiness is often communicated through an elaborate series of vocalizations and phonotactic responses (Duellman and Trueb, 1984). Typically, male frogs will vocalize, and females will move toward the male's call (phonotaxis). Both the call and the locomotor response are governed by hormonally-organized pathways that connect sensory receptors, brain, and response systems (Moore et al., 2005). The activation of these pathways during the breeding season is done in concert with the same mixture of hormones that organized these pathways. Precise hormone levels are critical to proper organization and activation of these reproductive pathways (Moore et al., 2005).

The African clawed frog, *Xenopus laevis* is unique among calling frogs in that both sexes actively vocalize. Calls are made when the laryngeal dilator muscles contract

and relax, quickly opening and closing the paired laryngeal arytenoid cartilages. The resultant ticking sound varies between the sexes in both call frequency and call amplitude (Kelley, 1986). Males can elicit three distinct call types: advertisement, amplectant, and growling. All three male-typical calls vary in frequency and oscillate in amplitude, and each surpasses ticking frequency and amplitude in female-typical calls. If a female is gravid, she will greet an advertising male with a rapid ticking call. Non-reproductive females produce a slow ticking call to deter a male's amplectant advances (Yamaguchi and Kelley, 2000).

Both the peripheral and central structures involved in procuring *Xenopus* calls show measurable sex differences. Structural and physiological sex differences in the *Xenopus* vocalization system extend to the level of the neuromuscular synapse. Motor output can be mapped from the laryngeal motor nucleus in the caudal medulla from which motor fibers exit through the most caudal root of the four-root cranial nerve IX-X complex (Kelley, 1986). These fibers, forming the laryngeal motor nerve, enter the thorax and synapse with the laryngeal dilator muscles.

During development, androgens organize the male vocalization system such that the somata in the laryngeal motor nucleus are larger and more numerous than those of the female (Kay et al,. 1999). The male and female peripheral vocal structures are also measurably different. The male *Xenopus* laryngeal nerve contains more axonal fibers than the female (Kelley and Dennison, 1990). Likewise, the laryngeal dilator muscles of the male consist of fast twitch muscle fibers, while there are only slow twitch fibers in the female larynx (Kay et al., 1999). Overall, the relative mass of the laryngeal cartilage and muscle is greater in the male than in the female. Distinct differences at the synapse drive

the male frog's ability to elicit an oscillating call. Because the male neuromuscular synapse is weak, it must be constantly modulated, resulting in amplitude differences among ticks. The female *Xenopus*' strong neuromuscular laryngeal synapse produces comparatively slow ticks of consistent amplitude.

Development of sexually different tissues depends on an embryonic critical organizational period when communication and reproductive structures are especially sensitive to cytotoxic agents and steroid hormones. Anatomical sex differences in *Xenopus laevis* brain are apparent by sexual maturity (6 months post metamorphosis), as are noticeable differences in laryngeal structure. Though not yet functional, these sexually different vocal structures are organized during the larval period to be activated after the frog reaches sexual maturity (Kelley, 1986). Because the developing systems are incredibly sensitive to the influences of chemical agents and steroids during the organizational period, any deviation from the necessary biochemical and hormonal levels could result in permanent morphological or physiological anomalies that may affect the frog's ability to successfully reproduce.

In the six months after metamorphosis, natural cell death solidifies the sex differences between male and female vocalization structures (Kay et al., 1999). Typically males lose nearly 25% or the vocal motor neurons (n. IX-X neurons), and females will lose nearly 47% of their n. IX-X neurons. Androgens, therefore, do not have a proliferative effect, rather the presence of androgens seems to protect neurons from scheduled cell death (Kay et al,. 1999). Furthermore, when treated with the androgen, dihydrotestosterone (DHT), female frogs had more n. IX-X neurons than untreated

females, so much so that at sexual maturity, treated females and untreated males showed no typical sex differences in n. IX-X neuron number (Kay et al., 1999).

Hormone levels are not only essential during development and organization. There exists other critical periods of hormone sensitivity during the adult frog's breeding period when sex hormones activate reproductive tissues essential for mating behavior and reproduction. Like the organization period, proper hormone levels are absolutely necessary for successful courtship, reproductive behaviors, and mating. Proper formation of the vocal structures during development will increase the likelihood of the male frog's ability to attract mates. Alterations to the developing vocal circuitry are thought to be more likely, owing to the animal's heightened sensitivity to biochemical influences. Changes in morphology after a frog reaches adulthood, however, are often developmentally constrained, because anatomy is usually fixed after the organization period (Moore et al., 2005). Even after exposure to exogenous levels of sex hormones, developmental constraints often limit any changes in morphology of the reproductive system. In *X. laevis*, morphology and physiology established by androgens is most likely unchangeable, while features procured by estrogens (female laryngeal neuromuscular synapse) are often reversible (Moore et al., 2005). Because reproductive behaviors are dependent upon accurate biochemical and hormone levels for proper function, exposure to exogenous cytotoxic agents or steroids could alter courtship behavior.

With at least two critical periods of hormone sensitivity in frogs, the potential for disruption of reproductive organ development and function is quite strong. Many agricultural, waste, and industrial chemicals are known to be cytotoxic, neurotoxic, and are putative endocrine disrupting chemicals (EDCs), because they alter natural hormonal

events and can permanently change morphology and behavior by disrupting development. Because the endocrine system plays a big part in mediating development, reproduction, physiology, and behavior, EDCs can seriously affect an exposed frog's survival and success. Hayes et al. (2003) showed that very low concentrations (0.1 ppb) the herbicide Atrazine could feminize developing male frogs. Evidence from field observations of atrazine-exposed leopard frogs showed a high incidence of pseudohermaphoditism and also had more subtle testicular abnormalities like incomplete maturation and testicular oocytes (Hayes et al., 2002). By exposing developing larval leopard frogs to comparable concentrations of Atrazine found in the field, Hayes et al. (2003) confirmed that Atrazine feminized the male frogs.

Atrazine, now ubiquitous in the environment, is only one chemical in a cocktail of environmental endocrine disruptors. Working in synergy, many of these pollutants are transformed from individually harmless chemicals to potent endocrine disruptors. There are several EDCs like Atrazine, however, that are individually as harmful. Polybrominated flame retardants (PBDEs) are as common in the environment as herbicides, pesticides, and industrial pollutants (Schechter et al., 2005). There are currently 209 different brominated flame retardant congeners that are often added to textiles, clothing, and electronic components to combustion. Environmental sampling studies have detected measurable, biologically active levels of PBDEs in not only landfill runoff, but in house dust, aquatic sediment, deep sea mammals, and even the blood plasma and breast milk of humans (Schechter et al., 2005). Like many endocrine disrupting chemicals, PBDEs are lipophilic and can be stored in body fat, making likely the possibility for transfer to offspring.

Organizational plan of the thesis

The following experiments show how PBDEs affect a critical period sensitive vertebrate system. The flame retardant, PBDE-209, an 8-bromine PBDE congener was chosen for its common industrial usage, and its relative ubiquity in the environment. PBDE-209, a flame retardant added to plastics and electronics, is one of the most commonly detected congeners in the environment (Schechter et al., 2005). A putative cytotoxin, neurotoxin, and endocrine disruptor, PBDE-209 is thought to induce apoptosis in brain cells as well as act as anti-androgenic agent (Costa and Giordano, 2007). Because the global goal of this experiment was to assay the effects of PBDE-209 on a hormone-dependent vertebrate system, I chose the vocal system of *Xenopus laevis*, the African clawed frog, as an ideal model. As previously mentioned, extensive studies of this model system's anatomy and physiology by the Kelley laboratory have provided the backdrop for the research described in this thesis. Based on these seminal studies, the present study centers on the hypothesis that PBDE-209 affects male *X. laevis* reproduction by altering its vocalization system.

Because PBDE-209 and the many other biologically active pollutants threaten reproduction and development, exposure studies like the present study are critical. Bioactive pollutants like PBDE-209 are so prevalent in the environment exposure may be constant beginning with mother handing down toxins to offspring and continuing through development to adulthood. This consistent exposure may disrupt the development of vocal tissues and behavioral pathways that could impair vocal system function resulting in reproductive failure. Following this introductory chapter, this work discusses the prevalence of PBDEs in the environment, their ability to disrupt behavior and

development, and the use of the *X. laevis* vocal system as a model to study PBDE-mediated disruption. Specifically my thesis discusses the effects of PBDE-209 exposure on the androgen-dependent vocal system, and whether the effects of PBDE-209 are restricted only to the developmental critical period. In the present study, two groups of frogs are exposed to PBDE-209 levels that are comparable to those found in the environment. Disruption by PBDE-209 during the developmental critical period is described by measuring PBDE-209's effects on sex ratio, growth, and vocal organ development in a group of *X. laevis* exposed solely during the sensitive critical period. The second group of frogs included PBDE-209 treated sexually mature adults, and the effects of PBDE-209 on vocal system anatomy and mate calling ability were measured. Changes mediated by the putative endocrine disruptor and neurotoxin, PBDE-209, can be measured by isolating the effects of PBDE-209 on both the development of the vocal organs as well as on the function of the vocal system.

The vocalization system is hormone-dependent during both critical periods of tissue organization and behavior activation. Exogenous chemicals, like PBDE-209, may interfere with both anatomy and function of any hormone-based system if cells are damaged and the availability of hormones and receptors is disrupted. After the experimental chapters, my concluding chapter summarizes PBDE 209's effects on both the developing and adult systems along with future directions for this research. With this work, I hope to elucidate the interactions between PBDE 209 and the two sensitive developmental critical periods in *Xenopus*, the organizational and activational phases, by assaying physiological change and the anatomical correlates of physiological change.

CHAPTER 2
THE *Xenopus laevis* VOCAL SYSTEM AS A MODEL FOR STUDYING THE PUTATIVE NEUROTOXIC AND ENDOCRINE-DISRUPTING CAPABILITIES OF THE BROMINATED FLAME RETARDANT, PBDE-209

Background

Vocal system development

Proper communication, essential for reproductive success between potential mates, relies on sex-specific development that is particularly sensitive to damage during critical periods, especially by endrocrine-mimicking pollutants. Proper communication increases reproductive capabilities between potential mates. Using mate calls or mating behaviors, an animal may advertise its interest in a mate or indicate its reproductive readiness. These mating behaviors depend on the development of sex-specific tissues that are organized during embryonic critical periods. Developing tissues are particularly sensitive to biochemicals, like hormones, during this critical period, requiring the precise levels of the body's own chemicals to properly organize peripheral tissues essential to mating behavior and their central nervous counterparts. Not only do endogenous chemicals influence developing tissues, but environmental pollutants may also affect these critical developmental periods. The neurotoxic and endocrine-mimicking capabilities of many environmental pollutants hinder proper development of tissues necessary for mating behavior and reproduction.

In the specialized mating behavior of *Xenopus laevis*, both sexes communicate interest and reproductive readiness through a series of vocalizations (Yaeger, 1984) that rely upon precise critical period development of vocal circuits and tissues that differ sexually. In the male, early in the developmental critical period, androgen receptor

production is detected in peripheral vocal organs, including laryngeal cartilage and laryngeal muscle cells, within the first two months post metamorphosis. At the same time, the vocal motor nucleus (n. IX-X) in the hindbrain begins to concentrate androgens (Wetzel et al., 1985). Androgen receptor production in the peripheral vocal structures and androgen concentration in the brain signal the beginning of vocal circuit organization. Androgen sensitivity in both peripheral and brain structures gives way to the formation of a hardy laryngeal cartilage and robust laryngeal dilator muscles that are built primarily of fast twitch fibers. The large male larynx provides an expansive synaptic field for the burgeoning laryngeal motor nerve to make many neuromuscular connections. Likewise, in the caudal medulla, the androgen-dense n. IX-X of the male maintains its high neuron population while growing more complex dendritic arbors that will eventually seek connections with central motor pattern generators (dorsal tegmental area of the medulla, DTAM, and reticular formation, RF) (Wetzel et al., 1985). The maintenance of n. IX-X number as well as the n. IX-X to DTAM and RF connections are all androgen mediated, are all unique to male *X. laevis*, and are all essential for sex-specific differences in mate vocalization. In contrast, in the female, androgen levels are low resulting in smaller laryngeal cartilages and muscle cells as well as n. IX-X with fewer and smaller neurons. Neuromuscular connections between the laryngeal nerve and the larynx are fewer in the female compared to the well-connected male vocal circuitry. Likewise, the connections from n. IX-X to afferent areas in the brain are also limited or absent in females because of the low levels of circulating androgens (Wetzel et al., 1985). The absence of male-level androgens during the critical period of development limits vocal circuit anatomy and the resultant female vocalizations.

In adult females and males, the sexually dimorphic vocal structures that develop underlie the differential behavioral capacities. In male frogs, both central nervous and peripheral laryngeal anatomy reflects the male's ability to procure rapid click vocalizations. Male-typical advertisement calls consist of rapid click vocalizations of oscillating amplitude (Tobias and Kelley 1995) made possible by sexually dimorphic vocal circuitry. Specifically, male laryngeal muscle, cartilage, laryngeal motor nerve, and motor nerve cell bodies in the hindbrain laryngeal motor nucleus (n. IX-X) are all measurably larger in males than in females (Wetzel et al., 1985; Tobias and Kelley, 1995; Simpson et al., 1986). Male vocal motor neurons are also more numerous and synapse weakly with the laryngeal muscle. Weak synapses necessitate constant modulation of the motor signal to the muscle, which results in the oscillating amplitude of the male click calls (Wetzel et al., 1998). In contrast, female click vocalizations are slower and of relatively short and similar amplitude compared to the elaborate male vocalizations because of vocal morphology constraints. Female click vocalizations are limited in amplitude and frequency, because the larynx is smaller and the laryngeal motor nucleus that serves as the vocal driving force is smaller and contains fewer neurons, resulting in female-typical vocalization.

These male and female differences arise through hormonally controlled processes during critical periods. The developmental critical period takes place during the six months after metamorphosis, marks a time when androgen release and increasing androgen sensitivity in developing male vocal tissues helps set up the vocal circuitry for male typical calls. For *X. laevis*, vocal tissues differentiate sexually during the six months following metamorphosis. This six month developmental critical period involves

intricate biochemical processes, some requiring the actions of sex hormones, that organize the areas of the brain in charge of mating vocalizations. This developmental critical period, signals a time when the male brain is especially sensitive to these biochemical processes, especially androgen-related actions that shape song circuit and motor patterning nuclei (Kelley, 1986).

During the critical period, both brain and laryngeal vocal structures in the frog are actively differentiated by androgens. At the start of the critical period, male and female *X. laevis* have similar numbers of vocal motor neurons in the cranial nerve glossopharyngeal-vagal motor nucleus, or vocal motor nucleus, n. IX-X (Kelley and Festamaker 1983). The nucleus n. IX-X, a motor nucleus, located in the caudal medulla, is androgen sensitive and endures many anatomical and physiological changes during the developmental critical period that later shape reproductive behavior. During the six months post-metamorphosis, the male n. IX-X concentrates androgens, and in the peripheral vocal organ, the larynx, there is a profound increase in androgen receptor population (Wetzel et al., 1986). Specifically, androgens maintain the high numbers of neurons in the male n. IX-X, while female *X. laevis*, by the end of the critical period, lose 40% of their vocal motor neuron population (Kelley 1986).

Like the males, the females also vocalize, but their vocalizations differ in accord with their different vocal organ anatomy. The female vocal organ anatomy reflects call limitations. Though the female also produces clicks, typical female calls are much slower than male calls. Instead of oscillating click amplitude, each female tick has relatively the same, small amplitude. The female laryngeal muscles, nerves, and laryngeal motor nucleus are comparatively smaller than the male, the smaller anatomy resulting in

smaller, and slower clicks. Laryngeal neuromuscular junctions in the female are governed by relatively strong synapses, giving rise to click vocalizations of similar amplitude (Wetzel et al., 1983).

In addition to the vocal organs themselves, central tissues that control vocalizations also develop sexually dimorphic forms during the same critical period. Central nervous tissues in males, specifically the vocal circuitry, begin concentrating androgens two weeks before metamorphosis is complete. With rapid development of the larynx underway by the onset of metamorphosis the male laryngeal motor nucleus (n. IX-X) in the hindbrain appears relatively similar to the female n. IX-X (Hannigan and Kelley 1986). The massive laryngeal development, however, encourages the n. IX-X neurons to increase afferent contact of vocal circuit components with n. IX-X. By PM6, male n. IX-X neurons have 270% more dendritic branching than female neurons (Kelley and Fenstamaker 1983). Motor output must also support the robust male larynx. Thus efferent contact between N. IX-X and the larynx is also extended to support the male larynx's large synaptic field (Hannigan and Kelley 1986).

At the end of the critical period, the differences in male and female central and peripheral structures are measurable. The laryngeal motor nucleus, n. IX-X, shows the most remarkable sex differences. The elongate motor nucleus extends bilaterally for about 1mm from the level of the glossopharyngeal-vagal cranial nerve, N. IX-X, insertion to just caudal of the first spinal roots (Wetzel et al. 1985). Song circuit nuclei are appropriately wired to accompany either the limited female larynx or the substantially larger male larynx. Absent in females are the robust afferent connections from the anterior parts of the song circuitry (preoptic area, POA, to the dorsal tegmental area of

the medulla (DTAM)), and collateral connections from the DTAM to the male n. IX-X. Also absent in females are the more numerous and larger cell bodies that occupy adult male n. IX-X (Simpson et al., 1986). By sexual maturity, coinciding with the end of the developmental critical period, the *X. laevis* n. IX-X contains the appropriate number of neurons for sex-specific mating vocalizations (Kelley, 2002). Sex-specific vocal behaviors reflect the architecture underlying hormone-sensitive structures.

For these sexually dimorphic changes, the levels and times of androgen expression are crucial. The male *X. laevis* vocal system is organized by androgens and is later maintained by androgens that also guide its function (Kelley, 1996). Production of a male advertisement call requires circulating androgens and an increase in androgen receptors in both larynx and brain structures (Kelley, 2002). Without these crucial levels of androgens, the developing male vocal system may not function properly, and the male will be unable to attract suitable mates. In contrast, females, whose vocal system anatomy is shaped in the absence of androgens, still can vocalize, but make comparatively less elaborate calls.

Chemical Pollutants and Development: Brominated Flame Retardants

The development of hormonally dependent tissues during the critical period may be altered by exposure to common environmental pollutants that skew the specific balance of hormones and disrupt development. Pesticides, such as atrazine or DDT, for instance, are known to disrupt gonad development in frogs during critical period exposure (Hayes et al., 2003). Polybrominated flame retardants (PBDEs) are also common pollutants that are known to act as neurotoxins and anti-androgens. They are ubiquitous in the environment, and are not only present in high concentrations in

pollutant runoff, but also in marine and fresh waters, soil, house dust, clothing, textiles, and household electronics. These flame retardants are so common in everyday items they are likely to be absorbed by humans. PBDEs are detected in human fat tissue and serum, and in high concentrations in human breast milk (Schechter et al., 2005). Because flame retardants have been detected in human breast milk, concern falls on the amount of transfer from mother to child and the effects of PBDEs on developing young.

PBDEs are relatively stable and persistent organic pollutants that are readily taken up into the body and are commonly found in the tissues of wildlife and humans (Schechter et al., 2005). Because brominated flame retardant congeners are incorporated into countless everyday items, clothing, and electronics, they are thus a constant presence as an environmental pollutant (Costa and Giordano, 2007). Of the 209 different PBDE congeners, the mostly widely used congener is PBDE-209 or deca-PBDE (Viberg et al. 2003). PBDEs are commonly incorporated into plastics, textiles, and building materials to slow or prevent combustion. PBDE-209, in particular, may be incorporated into commercial products, but manufacturing does not fix the PBDE into the product (Hutzinger and Thomas, 1987). Thus its chances of leaching into the environment are even greater. Once in the environment, PBDE-209 is thought to be relatively stable like other persistent organic pollutants, the polychlorinated biphenyls (PCBs). Like the PCBs, PBDEs are readily taken up into the body, and are now commonly found in the tissues of both aquatic and land wildlife and humans (Schechter et al., 2005).

Because brominated flame retardant congeners are incorporated into countless everyday items, clothing, and electronics, they are thus a constant presence as an environmental pollutant that is readily absorbed into the body (Costa and Giordano,

2007). PBDEs are detected nearly everywhere: water, soil, air, house dust, commercial food products, and household items (Darnerud et al., 2001; Schechter et al., 2006; Law 2006; and Chen et al., 2007). PBDE-laden pollutant run-off had been detected at extremely high levels (270-8400 μg/g dry weight = ppb) in sediment cores from the Western Scheldt River in Belgium (Covali et al., 2004), and levels in the livers of fish collected from Belgian watersheds ranged from 0.84-128 ppb in the Belgian North Sea, and 15-984 ppb in the Western Scheldt Estuary. PBDEs were also detected in high concentrations (0 – 212 ppb) from the San Francisco Bay (Oros et al., 2005), and up to 4600 ppb in the suspended particulate matter in the Netherlands' Western Scheldt River's water column (de Boer et al. 2003).

PBDEs taken up from the environment persist in fatty tissues later to be mobilized to offspring. PBDEs have been detected in wildlife and human body tissues, including animal blubber, liver, and serum (Darnerud et al., 2001; Schechter et al., 2006; Law 2006; and Chen et al., 2007). Environmental PBDE levels in North America are comparable to concentrations in Europe and Asia, and are readily bioavailable as a pollutant in both air and water (Costa and Giordano, 2007). United States women bear a body burden of PBDEs ranging from 4-419 ppb in their breastmilk (Schechter et al., 2005). In addition to the body burden, positive correlations have been made between high PBDE concentration in breast milk and incidents of testicular deformities in male offspring (Main et al., 2007). Since body burden can be linked to developmental deformities, determining the effects of PBDEs on developing offspring is crucial.

Fetal exposure in humans is mediated across the placenta and may alter the intricate processes involved in organ development. As organs become more defined and

sex differences are established in the fetus, the delicate balance of biochemical processes and hormone levels may be altered by exposure to chemical pollutants like PBDEs. Gestational PBDE exposure studies in rodents show persistent developmental effects on sex specific morphology and behavior that are evident even in adulthood (Lilienthal et al., 2006). Moreover, the detectable presence of PBDEs in human umbilical cord blood, maternal serum, and breast milk suggest that the developing human fetus is continuously exposed to flame retardant pollutants for the entire pregnancy, and the exposure continues if the child is breastfed (Guvenius et al., 2003). Positive correlations between PBDE levels in maternal breast milk and cryptorchidism (undescended testes) in male offspring (Main et al., 2007) show that PBDE exposure during human critical periods disrupts sex-specific tissue development. Because the height of human critical period development happens during the last trimester of gestation and continues through the first two years after birth, exposure to anti-androgenic and neurotoxic PBDEs puts the developing fetus at risk for long lasting developmental problems. PBDEs seem to target the development of sexually different morphological tissues and sex-specific behaviors suggesting disruption of critical period hormone levels, likely as a result of maternal transfer. Our bodies carry a large burden of pollutant chemicals, including PBDEs, which are likely handed down to our offspring. Thus it is crucial to determine how PBDEs interact with sensitive developing systems.

Neurotoxic, behavioral, and endocrine disrupting effects are common in developing animal models that are exposed to PBDEs (Legler 2008). Behavioral abnormalities are a common measure of developmental neurotoxicity, and often, PBDE-induced anomalies are known to worsen with age. Behavioral derangements such as

decreased habituation, hyperactivity, altered motor performance, decreased startle reflex and fear conditioning were common among PBDE-treated rodent models (Viberg et al., 2005; Kuriyama et al., 2005; Lilienthal et al., 2006; Rice et al., 2007). Specifically, PBDE-209, the flame retardant congener used in the present study, is readily taken up by early postnatal mice and distributed throughout the body, including into the brain (Viberg et al, 2005). If absorbed during early critical periods of brain development in mice, PBDE-209 induces neurotoxic effects that later, as adults, result in decreased habituation (Viberg et al., 2005). The inability for an animal to habituate to stimuli is a common indicator of neurotoxicity and may precede prolonged stress and hyperactivity. Neurotoxic effects, such as hyperactivity and decreased predator avoidance were also noted in PBDE treated killifish (Timme-Laragy et al., 2006).

During the critical period, hormone-dependent tissue development is also altered by PBDEs resulting in the disruption of sexually different tissues and behaviors. Anomalies in thyroid hormone levels in rats, for instance, were common after PBDE exposure. Examples of endocrine disruption include anomalies in levels of thyroid hormone in PBDE-209-treated rats (van der Ven et al. 2007). Disruption of thyroid hormones, which are involved in growth and metabolism, will severely harm development. PBDE's alteration of sex hormone related tissues further increases concern over developmental exposure to PBDEs. Specifically, when pregnant rats were treated with PBDE-209, male offspring showed anatomical changes in reproductive organs (reduced ano-genital distance) and changes male-typical behaviors (reduced sweet preference) that continued to be apparent into adulthood (Lilienthal et al. 2006). Significant levels of PBDE and its metabolites could be detected in rat placenta and

through transfer to embryos *in utero* (Kuriyama and Chahoud, 2003, Viberg et al., 2003). After birth, these chemicals were also detected in rat brain, sometimes affecting behavior as the animals grew into adults (Kuriyama et al., 2003).

Like mammalian hormone-dependent and critical period-dependent systems, the *Xenopus laevis* vocal system is sensitive to hormones during the developmental critical period, and thus provides an ideal model for studying how endocrine-mimicking pollutants may affect behavioral system neurodevelopment. In the present study, the *X. laevis* laryngeal motor nucleus (n. IX-X), serves as the model for assaying critical period anatomical changes in both developing and adult frogs treated with PBDE-209. Specifically, the goals of this experiment were to: 1) successfully and reliably identify n. IX-X, 2) determine if environmentally relevant levels of PBDE-209 has critical period-specific effects on developing frogs treated over the developmental critical period, as well as 3) treating adult frogs with environmentally relevant levels of PBDE-209, and 4) determining whether PBDE-209 alters vocalization ability in treated adults. Meeting these goals would also provide indications of PBDE-209's effects on both developing and adult systems.

Knowing that transfer to offspring and continued effects on the brain are a certainty, the study of PBDE-209's effects on the *Xenopus* vocal model will provide critical clues to the effects of PBDE-209 on both reproductive form and function. Developmental programs and hormone-dependent functions are often similar among vertebrates. Developing humans, like developing frogs, are not immune to the effects of endocrine disrupting chemicals like PBDEs. Because PBDEs can bioaccumulate in

adults and are passed to developing offspring, it is crucial to investigate how PBDEs interact with the process of embryonic development.

Because the vocal system of the male *X. laevis* relies upon precise biochemical and androgen-dependent developmental processes, it provides the ideal model for testing the effects of PBDE-209, a purported endocrine disruptor and neurotoxin. In order to investigate the effects of PBDE-209 on a developmentally hormone-dependent vocal system, *X. laevis* embryos are treated with PBDE-209 through the hormone-dependent critical period. PBDE-209 exposure may also affect hormone-dependent function of the vocal system. Thus the present study focuses not only on the effects of PBDE-209 on the developing vocal system, but also on PBDE-209's effects on the ability of the adult male *X. laevis* to vocalize.

CHAPTER 3
EXPERIMENTAL APPROACH: CRITICAL PERIOD AND NON-CRITICAL PERIOD EXPOSURE OF *Xenopus laevis* TO PBDE 209, EXPERIMENTAL DESIGN AND DATA ANALYSIS

In order to identify the laryngeal motor nucleus (n. IX-X) in the *Xenopus* hindbrain, several labeling and reconstruction methods were employed: 1) cresyl violet and 3D reconstruction, 2) nerve labeling with a retrograde, lipophilic dye (DiD), and 3) nucleus labeling (fluorescent Nissl stain) and confocal microscopy. After n. IX-X was clearly defined using these methods, adult male *X. laevis* were subject to 12 week's treatment with PBDE-209. Following treatment, confocal analyses of fluorescent-labeled image stacks were used to quantify n. IX-X neurons.

Animals

Because study animals required environments free from exogenous chemicals, *Xenopus laevis* (n = 20) were purchased from Xenopus Express (Homosassa, FL) then kept in the laboratory in aerated FETAX (Frog Embryo Teratogenisis Assay-Xenopus), a "pond-like" solution made from a distilled water base with added metabolic salts. Frogs were maintained in a temperature-controlled room (26˚C) with a 12-hour light: 12 hour dark cycle. FETAX was changed every third day, and frogs were fed a diet of NASCO (Fort Atkinson, WI) *Xenopus* brittle every other day (approximately 5 pellets per frog), and uneaten food and waste were removed each day by suction.

In order to properly preserve the brain tissue containing n. IX-X, frogs were euthanized by over-anesthetization in 0.1% tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO), and perfused transcardially with saline followed by 4%

paraformaldehyde (PFA). Brains were dissected from the frogs then fixed in 4% PFA in 30% sucrose for at least 24 hours before embedment and analysis. Precise dissection (1) revealed the approximate location of n. IX-X in the male *X. laevis* hindbrain and the architecture of the cranial nerve N. IX-X complex for which the 4th root serves as the conduit for vocal motor nerve to the larynx.

3D Reconstruction

Two representative brains were dissected from untreated, adult *Xenopus laevis* males so that three-dimensional reconstructions of the hindbrain could be made. Once dissected, tissues were subject to an alcohol dehydration series for embedment in paraffin. Paraffin-embedded brains were serial sectioned at 10μm on a rotary microtome. Tissues were then affixed to subbed slides, stained with cresyl violet, then analyzed using Neurolucida and Neuro Explorer software for the Nikon Eclipse E600 compound microscope.

First, a three-dimensional representation of the hindbrain was constructed using serial paraffin sections (10μm) accurate 3-D renderings of the hindbrain could be constructed. For each section, the brain's outline, ventricles, and nuclei were traced with representative colors using Neurolucida. The tracings were then imported into Neuro Explorer and bundled together to create a 3-D representation of the hindbrain from which n. IX-X nucleus depth and length could be determined.

Atlas Diagrams

Hindbrain structures and nuclei in the male *X. laevis* brain were mapped by constructing atlas diagrams from 10 μm serial hindbrain sections. The locations of hindbrain nuclei were determined by referring to *X. laevis* vocal anatomy studies by

Simpson et al. (1986) and Wetzel et al. (1985). A representative control brain from an adult male *X. laevis* was processed for paraffin embedment and serial sectioned at 10 μm. Photomicrographs (10x) of every twentieth section of the hindbrain were taken using Neurolucida in conjunction with a Nikon Eclipse E600 compound microscope. Because the sections were too large to capture in one photomicrograph, portions of the sections were taken. Photomontages of these portions were then made using Calico software by Kekus. Using Corel Draw 11, the montages were cropped by removing the left side of the brain from the dorsoventral midline to the distal edge of the left side of the brain. Background artifact and meninges were also removed, and outlines of the right side of the brain and important structures were made. The outlines and structure tracings were inverted and transposed to the right sides of the representative figures.

Retrograde and Fluorescent Nissl Labeling

The remaining male *X. laevis* control brains were dissected, taking care to include the four root cranial nerve IX-X complex that contains the motor nerve to the larynx. Two brains were processed for retrograde labeling. For these brains, the fourth and caudal-most root of the complex was injured and implanted with a small crystal of DiD, a slow-moving lipophilic, retrograde dye. The dyed tissues were placed in 0.2 % phosphate buffered saline and 4% PFA then allowed three to six months to incubate. After six months, the tissues were embedded in gelatin then cryo-sectioned (100 μm) horizontally on a Leica sliding microtome. Tissues were counterstained with Neurotrace 480 and analyzed on the Nikon Eclipse TE-2000U confocal microscope and images were rendered using Nikon EZ-C1 software.

The remaining brains were also serial cryo-sectioned at 100 μm along the horizontal plane and positioned on glass slides. Brains were usually 1200 μm thick, with the highest concentration of n. IX-X cell bodies located at a depth of 600-700 microns from the dorsal surface of the brain. Tissues were stained with Neurotrace 480 (Invitrogen), a fluorescent Nissl stain, to help locate the laryngeal motor nucleus (n. IX-X), and then viewed using a Nikon Eclipse E600 confocal microscope. The tissue section with the highest n. IX-X cell body concentration was magnified to 20x, and a 100μm image stack was collected. For each image stack, neuron number was quantified by counting each cell body for which only the nucleolus could be seen, and cell body diameter was measured along the longest plane.

PBDE-209 Exposure--Juveniles

To ensure that experimental animals were kept free from exogenous chemicals early stage Xenopus embryos (3-5 days post hatch) from multiple clutches were obtained from Xenopus Express (Homassasa, Florida). Once acclimated in the laboratory, embryos (200) were pooled then randomly separated into 5 tanks. Embryos from each tank were then randomly distributed into four two-gallon tanks. Each two-gallon tank was fit into a flow-through rack system for the duration of the experiment. At the beginning of the treatment period, the experimental set-up consisted of 20 two-gallon tanks, each containing ten tadpoles. Animals were maintained in the flow-through set-up and treated for the duration of the larval period through six months post-metamorphosis (approximately 8 months).

Flow-through system.

PBDE-209 seems to be relatively stable in the environment. PBDE-209 is not water soluble, rather it exist as particulate swept into water column that may be taken up by the body if exposed (Birnbaum and Stastkal, 2004). PBDEs were detected in high concentrations (0 – 212 ppb) from the San Francisco Bay (Oros et al., 2005), and up to 4600 ppb in the suspended particulate matter in the Netherlands' Western Scheldt River's water column (de Boer et al. 2003). Since environmental concentrations were so variable, our exposure concentrations were limited to the low end of what was found in the water column.

Five 30-gallon HDPE barrels were loaded with a stock solution of FETAX and PBDE 209. PBDE-209 was dissolved in a vehicle of 100 µl DMSO before being added to the FETAX solution. The experimental solutions were based on those found in nature. Surveys of estuarine fish and mussels showed levels ranging from 0 to 984ppb (Voorspoels et al., 2003). Levels in human subjects ranged from 0.01ppb to 419ppb (Darnerud et al. 2001, Schechter et al., 2005). Actual experimental PBDE-209 concentrations were quite conservative: 0 ppb, 0.1 ppb, 10 ppb, and 100 ppb PBDE-209 (Table 1). Larvae were raised in experimental solutions six months beyond metamorphosis (about 8 months total) to ensure sexual differentiation of n. IX-X brain tissues.

In order to minimize the interaction of other suspected endocrine disrupting agents with the experimental animals, a self-built flow through system was constructed, avoiding as much plastic and PVC material as possible. Commercial flow through systems are generally constructed with plastics and PVC piping. Plastics and PVCs are

Table 1. Juvenile *X. laevis* treated with PBDE-209

Treatment Group	# Larvae @ Start of Treatnemt	# Frogs After Treatment	
		M	F
Control = 0 ppb PBDE-209	40	7	14
Low Dose = 0.1 ppb PBDE-209	40	11	17
Middle Dose = 1.0 ppb PBDE-209	40	6	11
High Dose = 10.0 ppb PBDE-209	40	14	23

said to contain bioactive chemicals that disrupt endocrine function by acting as anti-androgens or estrogen mimics (Colborn et al. 1996). Because it was necessary to avoid interactions of other endocrine disruptors with the developing *X. laevis* vocal system, the flow-through system was made of glass, silicone, and high density polyethylene (HDPE).

The flow-through system (Figure 1) was set up such that each tank would receive solution at a rate of 20 ml/min. Solution was pumped through silicone tubes up to a glass box from which solution was diverted into four different tanks. Flow rate was controlled by Teflon-coated PTFE stopcocks that connected the glass box and the experimental tanks via silicone tubing and glass piping.

Because the half-life of PBDE 209 in water was about two days, another dose of stock PBDE solution was added every third day to the appropriate barrel for dosage. Solutions barrels required total replenishment approximately once each month. Effluent was collected and aggravated charcoal was added to bind PBDE that had not yet

Figure 1. Flow through system built to deliver 25 ml/min of BDE-209 treated solution to tanks containing 10 tadpoles each. Concentrated stock was added to each large barrel and pumped to a glass distribution tank on top of the rack system. Silicone hoses were fitted with Teflon-coated PTFE stopcocks (Corning) that apportioned solution to the tanks at the appropriate rate. Over flow was brought back to solution barrels via silicone hoses, and effluent runoff from experimental tanks was pooled, treated with charcoal, and allowed to evaporate. Charcoal and evaporated effluent was disposed of by University of Miami Environmental Health and Safety Office according to OSHA regulations. Treatment lasted approximately 8 months through the developmental critical period to sexual maturity.

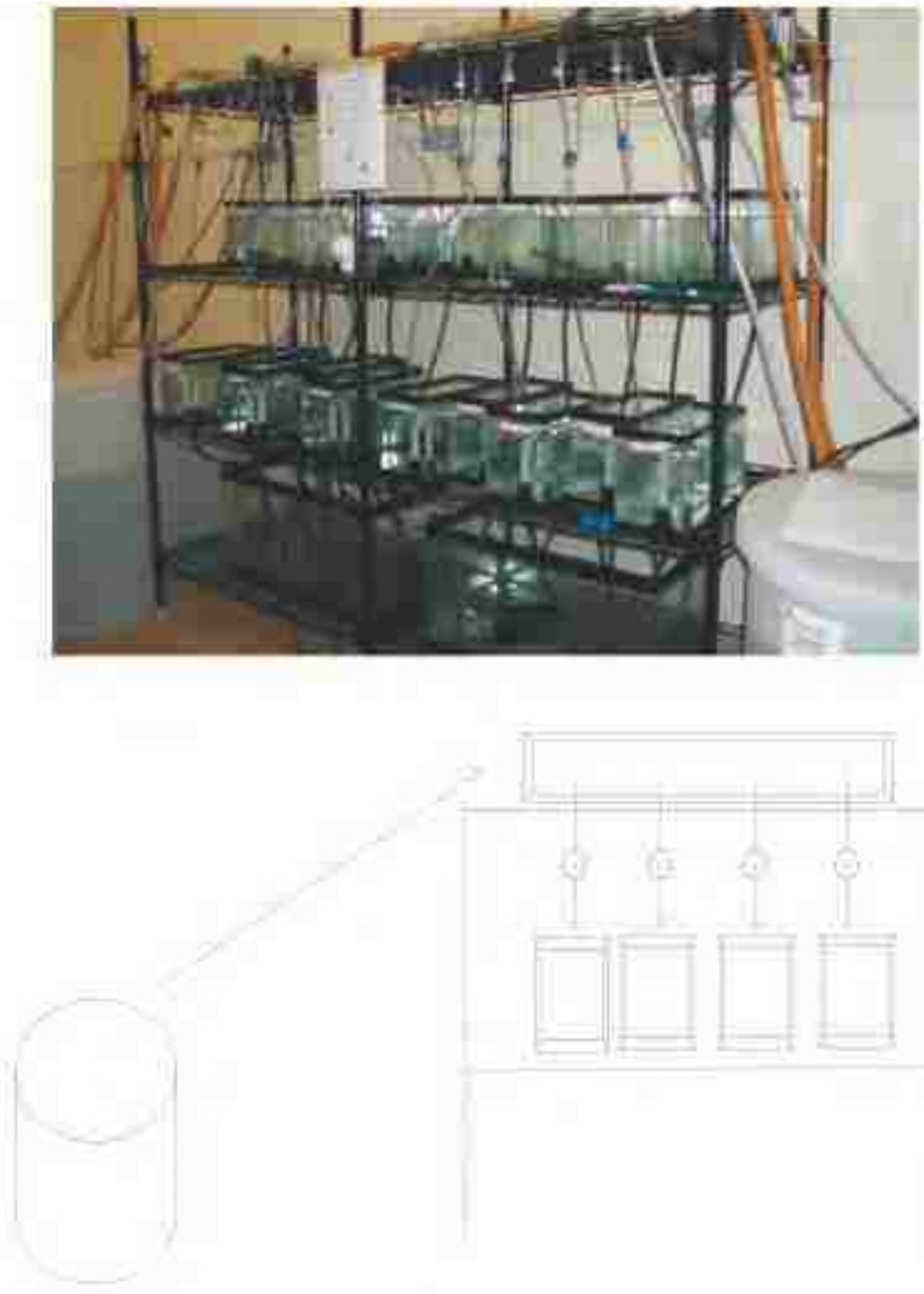


Figure 1

metabolized. Charcoal-treated effluent was left to evaporate under a fume hood each week. Both solid and liquid wastes were collected and disposed of according to University of Miami Environmental Health and Safety regulations.

Tissue staining and analysis

Once the animals reached sexual maturity, they were euthanized by over-anesthetization in 0.1% tricaine methane-sulfonate (MS-222) solution, weighed, and snout-vent lengths (SVL) were measured. The frogs were decapitated, and sex was determined by gonad dissection. In order to determine if the growth of the frogs was affected by PBDE-209 treatment, SVL and weight was compared among treatment groups using a one-way ANOVA. A chi-square test (alpha < 0.05) was also used to determine if there was an effect of PBDE-209 treatment on the sex ratio of surviving frog.

In order to properly preserve the vocal motor tissue in the brain, the frogs' heads were placed in 4% paraformaldehyde (PFA) solution in 30% sucrose for a minimum of 24 hours before brain dissection. Once brains were dissected, they were fixed in 4% PFA in 30% sucrose for a minimum of 24 hours. Tissues were embedded in gelatin and 30% sucrose and allowed to cure in 4% PFA and sucrose overnight. Horizontal cryo-sections (100 μm) of the hindbrain were made on a sliding microtome, placed on glass slides, and stained with Neurotrace (Invitrogen, Carlsbad, CA), a fluorescent Nissl stain.

Tissues were analyzed using EZ-C1 software for the Nikon Eclipse TE2000-U confocal microscope. The hindbrains were generally 700-800 μm thick, and the paired vocal motor nuclei (n. IX-X) ran laterally from the level of the obex to just rostral of the first spinal roots at a depth of 400-500 μm. Image stacks were collected using EZ-C1,

and n. IX-X neurons were counted in a single 100 μm image stack by scrolling through each stack and numbering only somata for which the nucleus was visible. A one-way ANOVA compared cell counts among surviving males, and sources of variation among treatments were determined using a Neuman-Keuls multiple comparisons post hoc test ($\alpha = 0.05$). Cell body lengths and widths were also measured and compared using a one-way ANOVA. The width and length of the entire n. IX-X nucleus was also measured and analyzed for variance among treatments.

PBDE-209 Exposure--Adults

Adult male *Xenopus laevis* (N = 75) were purchased from Xenopus Express (Homosassa, FL) and acclimated in the laboratory before being randomly distributed among ten glass aquaria (8-10 frogs per 20 gallon tank). All frogs were kept in 20 gallons of aerated FETAX (Frog Embryo Teratogenisis Assay-Xenopus), a "pond-like" solution made from a distilled water base with added metabolic salts, that was changed every third day to maintain sanitary chemical free conditions. If a frog died during the treatment period, it was immediately removed and the tank was cleansed and refilled. Frogs were fed a diet of NASCO (Fort Atkinson, WI) *Xenopus* brittle every other day (approximately 5 pellets per frog), and uneaten food and waste were removed each day by suction.

The male frogs were subject to one of four different treatments (Table 2) for a 12-

Table 2. Adult *Xenopus laevis* treated with BDE-209

Treatment	# Frogs @ Start	# Frogs Surviving
0 ppb BDE-209 (0.01 ml DMSO/L vehicle control)	20	16
0.1 ppb BDE-209	18	5
1.0 ppb BDE-209	18	4
10 ppb BDE-209	18	10

week period. PBDE-209 is not soluble in water, has limited solubility in ethanol, but goes readily into solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Saint Louis, MO). PBDE-209 was dissolved in DMSO to make concentrated stocks such that each tank would receive the same amount of DMSO (0.01 ml DMSO/L FETAX) but appropriate concentrations of PBDE-209. Concentrated stock was added to the FETAX solution after each water change. Control tanks (0ppb PBDE-209), served as vehicle controls and also received 0.01ml DMSO/L FETAX.

Vocal elicitation

Both male and female frogs were injected with human chorionic gonadotropin hormone (HCG; Sigma, St. Louis, MO) in order to spur the onset of the breeding condition and call elicitation. Males were given 600 IU HCG (following Yamaguchi and Kelley, 2000), and females received 800 IU. The frogs were kept in isolation tanks until the injected female was placed in the recording chamber with the male to help stimulate the male to vocalize. EMG and vocal recordings were done on free-swimming frogs. After recording, experimental frogs were euthanized for anatomical study.

Electrophysiology

Several hours after HCG injection, the male frog was anesthetized in a 0.1% tricaine methanesulfonate (MS-222) solution. While the frog was being anesthetized, a bipolar electrode was fashioned from a looped 50 μm diameter length of formvar insulated nichrome wire (A-M Systems; Carlsborg, WA) that was threaded through a 27-gauge needle. The loop was snipped, and 0.1 mm of formvar insulation was scraped from the ends of the cut wire to ensure proper contact with the laryngeal muscle fibers.

The wires protruding from the needle were then fashioned into small hooks, completing the bipolar electrode.

Surgical procedures follow Yamaguchi and Kelley (2000). The anesthetized frog was placed ventral side up, on a dish of ice. A small, 2 cm skin incision was made beginning at the area just caudal to where the underside of the arm meets the body, then the incision continued toward the midline. The underlying muscles were also cut and reflected back so that the laryngeal dilator muscle could be clamped and gently extruded from the incision site.

The electrodes were implanted deep into the belly of the laryngeal dilator muscle (Figure 2) and the wires were sutured to the laryngeal cartilage wing. The incision site was closed, making sure the electrode lead wires remained implanted in the muscle and extended out of the incision site. The frog was allowed to recover in a small isolation tank, and after a minimum of six hours, the lead wires were soldered to pins. The frog was then placed with an HCG-injected female in a recording chamber fashioned from a clear, polycarbonate shoebox. Most male frogs recovered quickly and began to swim within 15 minutes after surgery, and many began calling even before placement in the recording chamber.

Figure 3 shows a schematic of the EMG set-up. The electrodes were connected to the A and B inputs on a head stage and the differential inputs were amplified with a WPI DAM-70 preamplifier (Low pass filter = 300 Hz, High pass filter = 10 Hz, Gain = 10,000 X). The signal was filtered through a 60 Hz notch filter, then converted to a digital signal with aDI-720 USB A-D converter. EMGs were recorded using the WinDAQ software

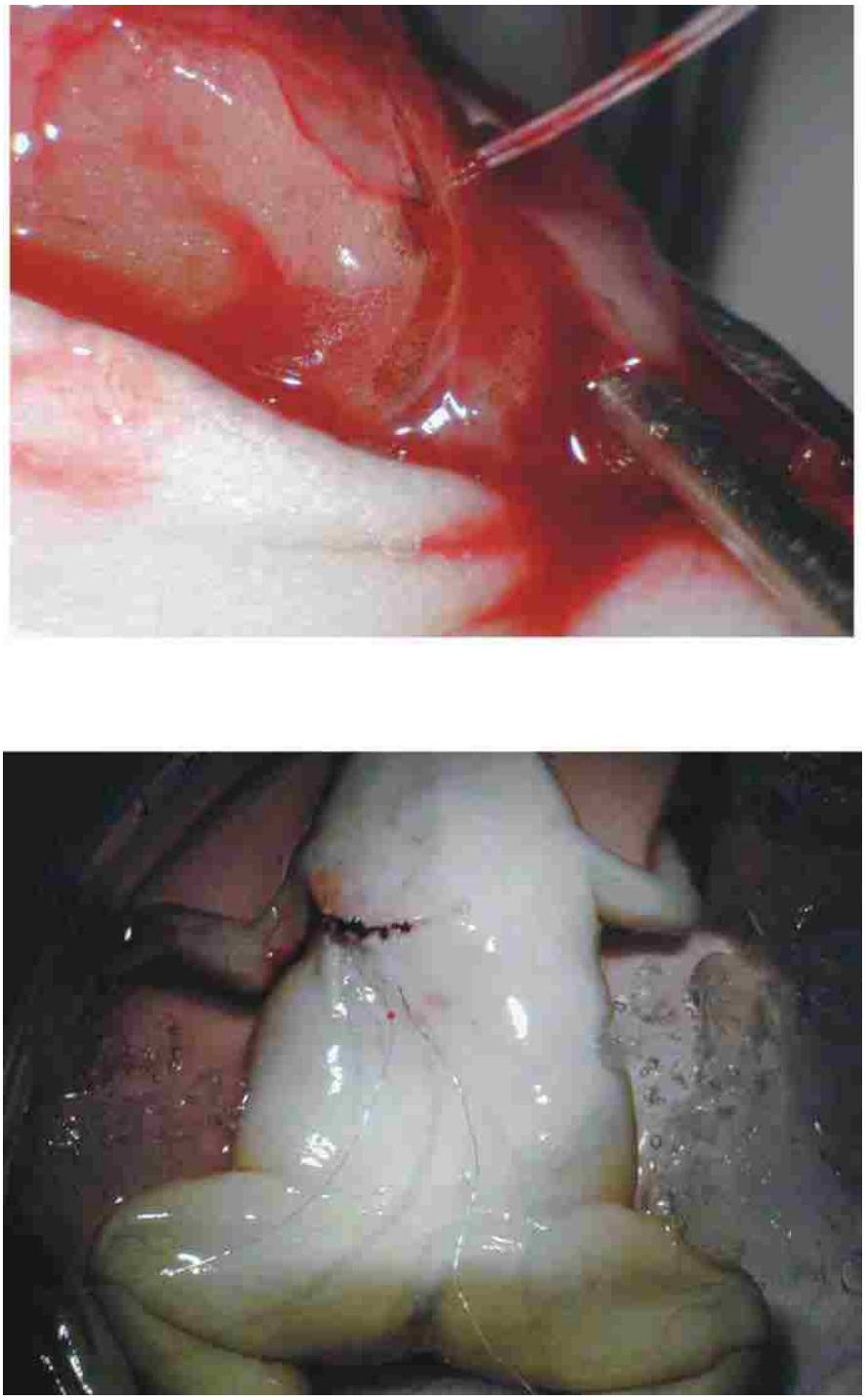


Figure 2. Photographs of bipolar electrode for recording EMG. Top photo shows electrode protruding from the belly of the laryngeal dilator muscle. Bottom photo shows the sutured frog post surgery with electrode lead wires exiting the incision site.

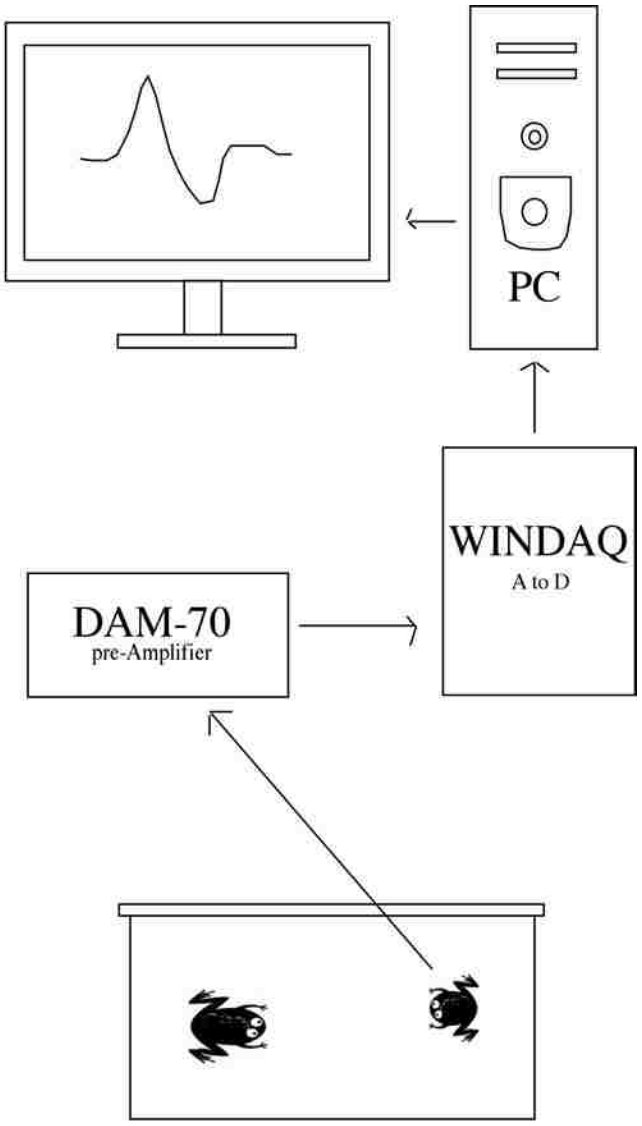


Figure 3. Schematic of EMG recording apparatus. Vocalizations were recorded as laryngeal dilator muscle contractions from free-swimming male *X. laevis*. Recording electrodes were connected to a head stage. The stage was connected to a DAM-70 pre amplifier where the signal was amplified, then converted to a digital signal by the WinDAQ USB converter then analyzed on a PC using WinDAQ software (DATAQ Instruments).

for PC. Signals were recorded at a sampling rate of 0.05 seconds/division and amplitude range of +/- 3000 μV. Some vocal recordings were also made using a hydrophone and Garageband software for Macintosh.

Statistical analyses—EMG

Calls were recorded for a minimum of three hours, with most recording periods lasting more than six hours. Call parameters measured for each frog included: 1) number of calls per recording period, 2) average call duration, and 3) average click amplitude per call (Figure 4). Since many calls contained smaller episodes or bursts, separate calls were determined to be three or more seconds apart. Call duration, therefore, included the entire calling episode including the smaller bursts. The recording was compressed 100 X (sampling rate = 0.5 seconds/division) in order to count the number of calls per treatment, taking care to recognize individual calls versus burst episodes within calls. For amplitude measurement, recordings were uncompressed (0.005 seconds/division) in order to accurately count amplitude of signal minus background noise. The ten highest clicks per call were quantified. Differences in call performance parameters among treatment groups were assessed with a one-way ANOVA ($\alpha = 0.05$). Specific differences between treatments were detailed with t-tests ($\alpha = 0.05$). Within treatment variation was also assessed for click amplitude. After EMG recordings, frogs were euthanized in 0.1% tricaine methanesulfonate (MS-222) and perfused transcardially with saline followed by 4% paraformaldehyde (PFA). Brains were removed and fixed in refrigerated 4% PFA in 30% sucrose. After 24 hours, brains were embedded in gelatin in 30% sucrose and post-

Figure 4

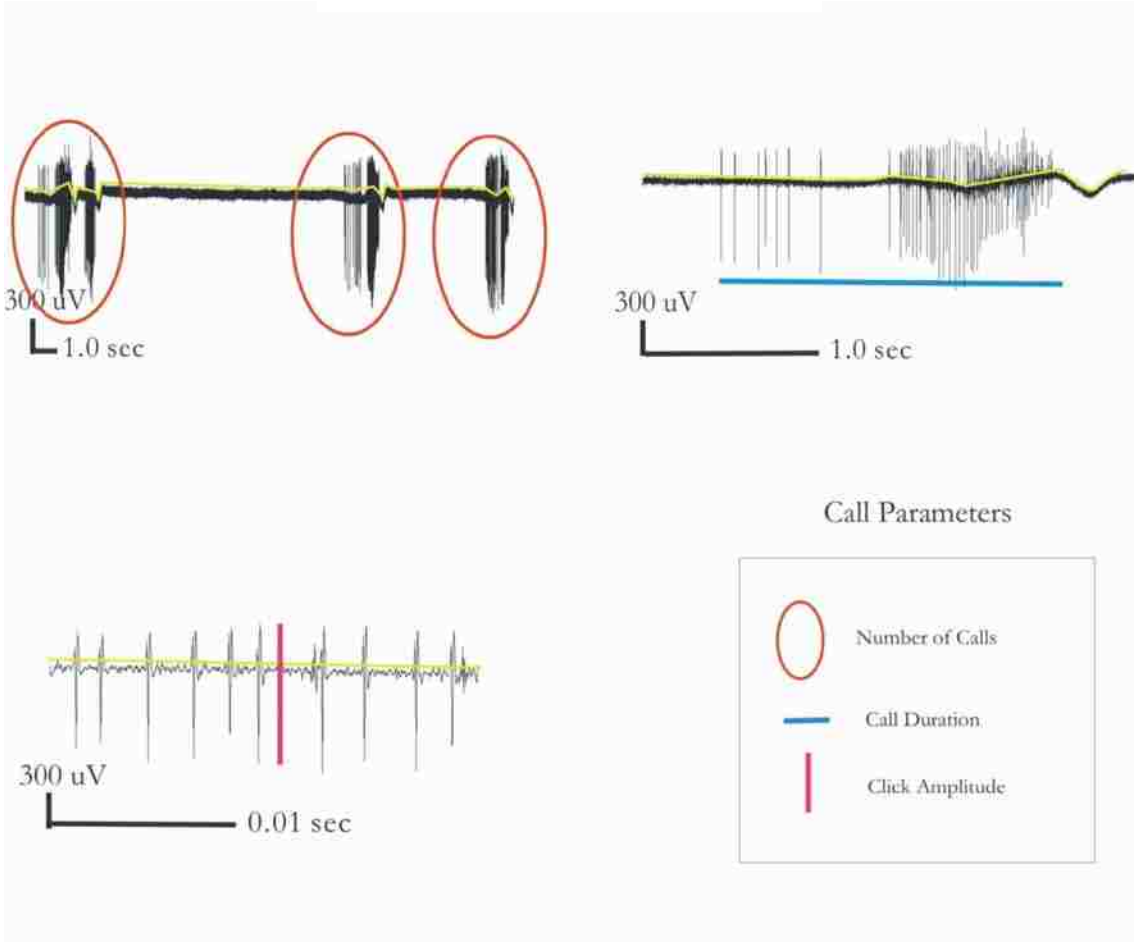


Figure 4. Call parameters measured included the counting the number of calls (red circle), calculating the duration of a call (blue line), and quantifying amplitude (magenta line). Recordings were first compressed (1.0 seconds/division) in order to count the number of calls per hour. Calls were differentiated from one another by having three or more seconds of silence between each burst of sound. The recording could be slowed to 0.1 sec/div in order to accurately measure the duration of each call. Finally, by uncompressing each recording to 0.01 sec/div, the amplitude of each click vocalization could be determined by measuring the height of each click above and below the noise (yellow line).

fixed overnight in 4% PFA in 30% sucrose. Post-fixed tissues were serial sectioned in the horizontal plane at 100μm on a Leica sliding microtome, stained with Neurotrace 480 (Invitrogen), then analyzed using EZ-C1 software for the Nikon Eclipse TE-2000U confocal microscope.

Most hindbrains were 1200-1500 μm thick, and the 300-400 μm thick laryngeal motor nucleus was located 600-800 μm below the brain's dorsal surface. Once n. IX-X was located at 4x magnification, the area was magnified to 20x so that the number of neurons could be counted accurately from an image stack. Image stacks (100 μm) were captured and analyzed using Nikon EZ-C1 software for the confocal microscope. Neuron cell bodies were counted by scrolling through the z-plane of a 100 μm image stack, and numbering only somata for which the nucleoli were visible. Each image stack was taken from the middle, and most populous section through the nucleus. Neuron counts were compiled for all surviving frogs. Because there were insufficient numbers of survivors in the low (0.1 ppb) and middle (1.0 ppb) cell counts were compared between only the control (0 ppb) and high dose (10 ppb) frogs. Somatal diameters were also measured, and average diameters were compared between control and high dose-treated frogs. Statistical comparisons of neuron number and somata length and width were made using one-way ANOVAs followed by Newman-Keuls multiple comparisons post-hoc tests with alpha set at 0.05. The entire n. IX-X nucleus was also measured and analyzed for variance among treatments.

CHAPTER 4
ANATOMY OF THE *Xenopus laevis* VOCAL MOTOR NUCLEUS

The vocal motor nucleus, n. IX-X of *Xenopus laevis* is located in the caudal medulla of the hindbrain. The male frog's n. IX-X is larger and more highly connected to afferent input areas and vocal patterning centers. As mentioned in chapter 2, the entire male vocal system is larger and more complex than the female vocal system in order to procure the male's more elaborate advertisement calls. The anatomical studies that follow were done in order to reliably locate n. IX-X for quantifying the laryngeal motor neurons in PBDE-209 treated *X. laevis.* Several methods were employed to illustrate n. IX-X's location and structure.

The male *Xenopus laevis* laryngeal motor nucleus (n. IX-X) is organized, maintained, and its function governed by androgens. In order to attract mates, a male elicits rapid click vocalizations of varying amplitude, vocalizations that reflect its robustly built vocal system. Located in the hindbrain, the male n. IX-X is densely populated with large cells receiving input through extensive dendritic arbors. Signals from n. IX-X travel on numerous fibers that exit the caudal medulla through the 4^{th} root of the cranial nerve N. IX-X complex to the laryngeal dilator muscles. Figure 5 shows the approximate location of n. IX-X in the dissected hindbrain of an adult male *X. laevis*. Both peripheral and central nervous structures rely heavily on precise levels of androgens for proper vocalization. Without critical androgen levels, the organization, up-keep, and function of the vocal system would not be possible. Anatomical study of the *X. laevis* hindbrain followed Kelley, 1997; Kelley et al., 1998; Simpson et al., 1986; and Wetzel et

Figure 5

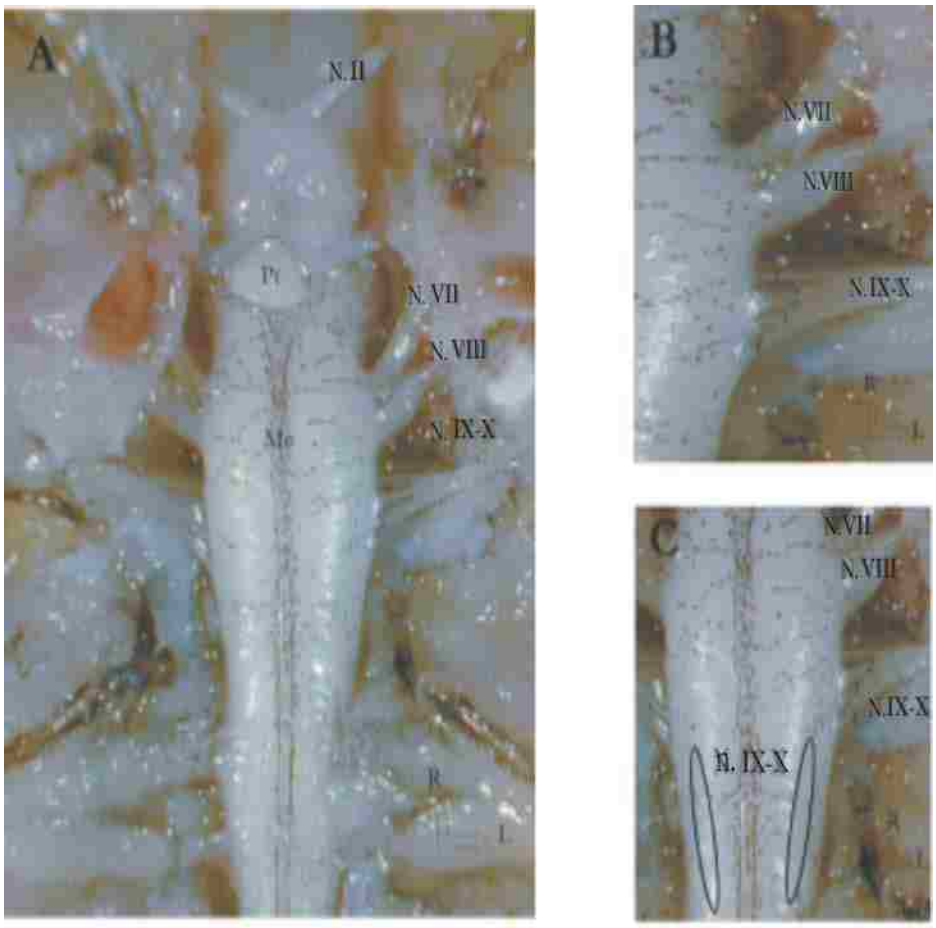


Figure 5. Gross dissection of the ventral side of the hindbrain of an adult male *X. laevis*. A. Full view of ventral brain from the optic nerve (N. II) to the spinal cord area. Pt. = pituitary, N. VII = facial nerve, N. VIII = vestibulocochlear nerve, N. IX-X = glossopharyngeal vagal nerve complex, ME = medulla. B. Close-up of 4-root glossopharyngeal-vagal nerve complex of which root 4 serves as the conduit for the laryngeal motor nerve. C. Lines encircle the approximate areas of the glossopharyngeal-vagal motor nucleus (n. IX-X) in relation to N. IX-X.

al., 1985 as anatomical guides. Line representations (Figure 6) and 3D reconstruction models (Figure 7) illustrate the location and dimensions of some hindbrain structures and n. IX-X in the caudal medulla. One of the vocal motor pattern generators, the pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM), serves as n. IX-X's primary afferent input. The other major vocal pattern generator, the reticular formation (RF), is a nucleus that runs medial to n. IX-X (Zornik and Kelley, 2007). As androgens shape the developing male nucleus many, intricate collateral connections are established between n. IX-X neurons and RF, connections that are not as robust in the female (Kay et al, 1999).

Atlas diagrams (Figure 8) of transverse sections through the caudal medulla illustrate a clearer picture of the nuclei occupying the male *X. laevis* caudal medulla and highlight n. IX-X. The cell bodies comprising n. IX-X are quite large, and they occupy paired columns that run laterally along the caudal medulla from the N. IX-X insertion to caudal of the obex and just anterior of the first spinal roots (Zornick and Kelley 2007, 2008). Cell bodies of n. IX-X were reliably located 600-800 μm from the dorsal surface of the hindbrain. The cells of the RF, the pattern generator governing vocalization, run adjacent to n. IX-X.

Fluorescent staining and retrograde dye tracing also provided clear pictures of n. IX-X's location in the hindbrain (Figure 9). Confocal analyses of fluorescent Nissl-stained hindbrain sections provided the most simple and reliable means for identifying and quantifying n. IX-X neurons, and these methods were used to analyze n. IX-X in PBDE-209 treated juvenile and adult frogs.

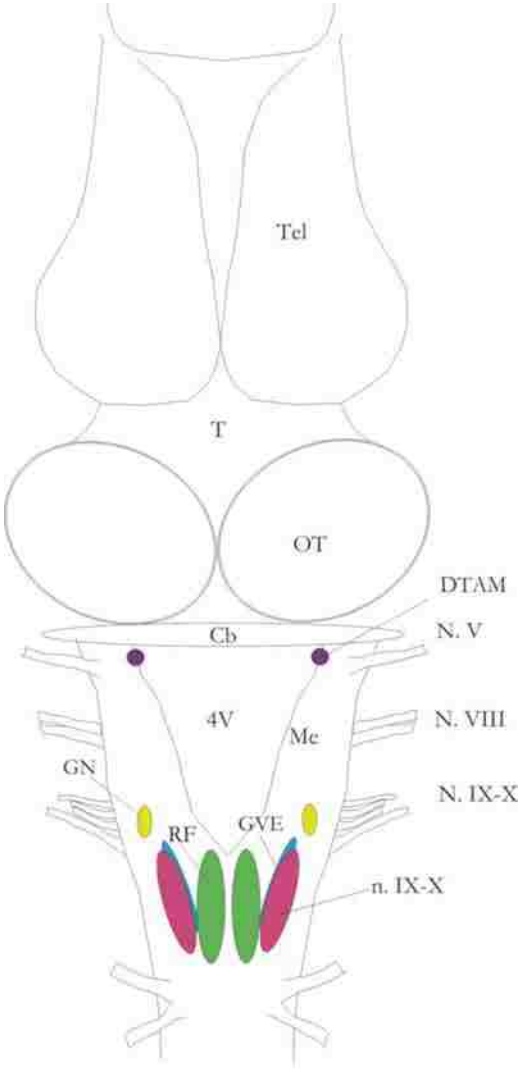


Figure 6

Figure 6. Line representation of the dorsal surface of the brain of *X. laevis* and highlighting the location of several important nuclei in the vocal circuitry. Cb = cerebellum, DTAM = dorsal tegmental area of the medulla, GN = glottal nucleus, GVE = general visceral efferents, N. V = trigeminal nerve, N. VIII = auditory nerve, N. IX-X = glossopharyngeal –vagal nerve complex, n. IX-X = laryngeal motor nuclueus, T = thalamus, Tel = telencephalon, 4V = fourth ventricle.

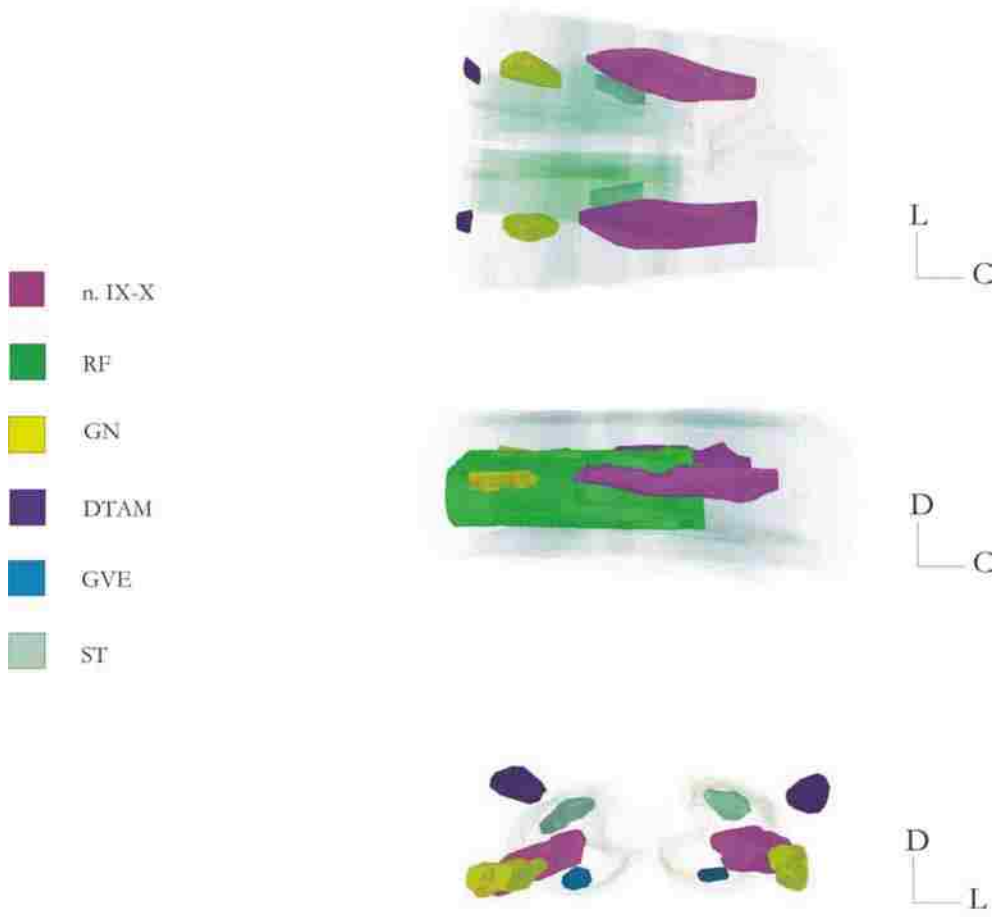


Figure 7

Figure 7. 3D representations of the hindbrain of male *X. laevis.* A. dorsal view. B. side view. C. anterior view. Representations were made by outlining neuroanatomical structures in 10 μm transverse serial sections through the hindbrain of a male *X. laevis.* Image stacks were combined with Neurolucida software and processed into 3-dimensional representations with NeuroExplorer software. Magenta colored areas represent n. IX-X and its close association with the vocal motor pattern generator, RF = reticular formation (green). Other labeled nuclei include: ST = solitary tract, DTAM = dorsal tegmental area of the medulla (primary afferent connection to n. IX-X), GN = glottal nucleus, GVE = general visceral efferents.

Figure 8 (1-3). Atlas diagrams from 10 μm serial sections through the caudal medulla of a male *X. laevis.* Tissue sections were stained with cresyl violet and viewed with a Nikon t-2000 compound microscope. Photomantages of the 20x photomicrographs were compiled using Calico software (Kekus). A. Line representation of the brain of *X. laevis*. Red lines represent sections (anterior to posterior, B-D) through the hindbrain. 6-1. Most rostral section through hindbrain. 6-2. As sections move caudally, n. IX-X somata appear and are more concentrated. 6-3. Most caudal sections through n. IX-X in the caudal medulla. GVE = general visceral efferents, n. IX-X = laryngeal motor nucleus, RF = reticular formation, ST = solitary tract, 4V = fourth ventricle. Black lines = 1mm.

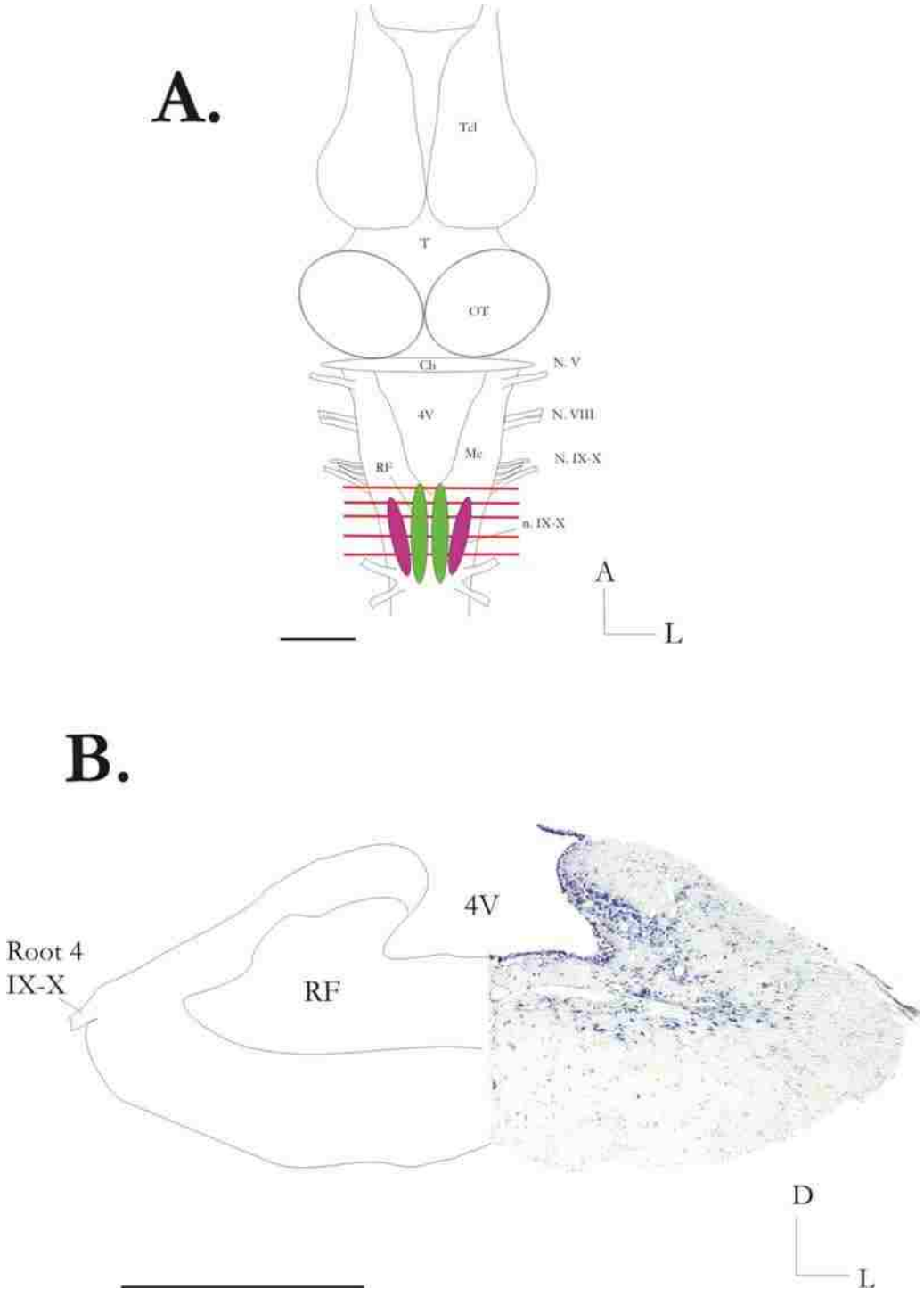


Figure 8-1

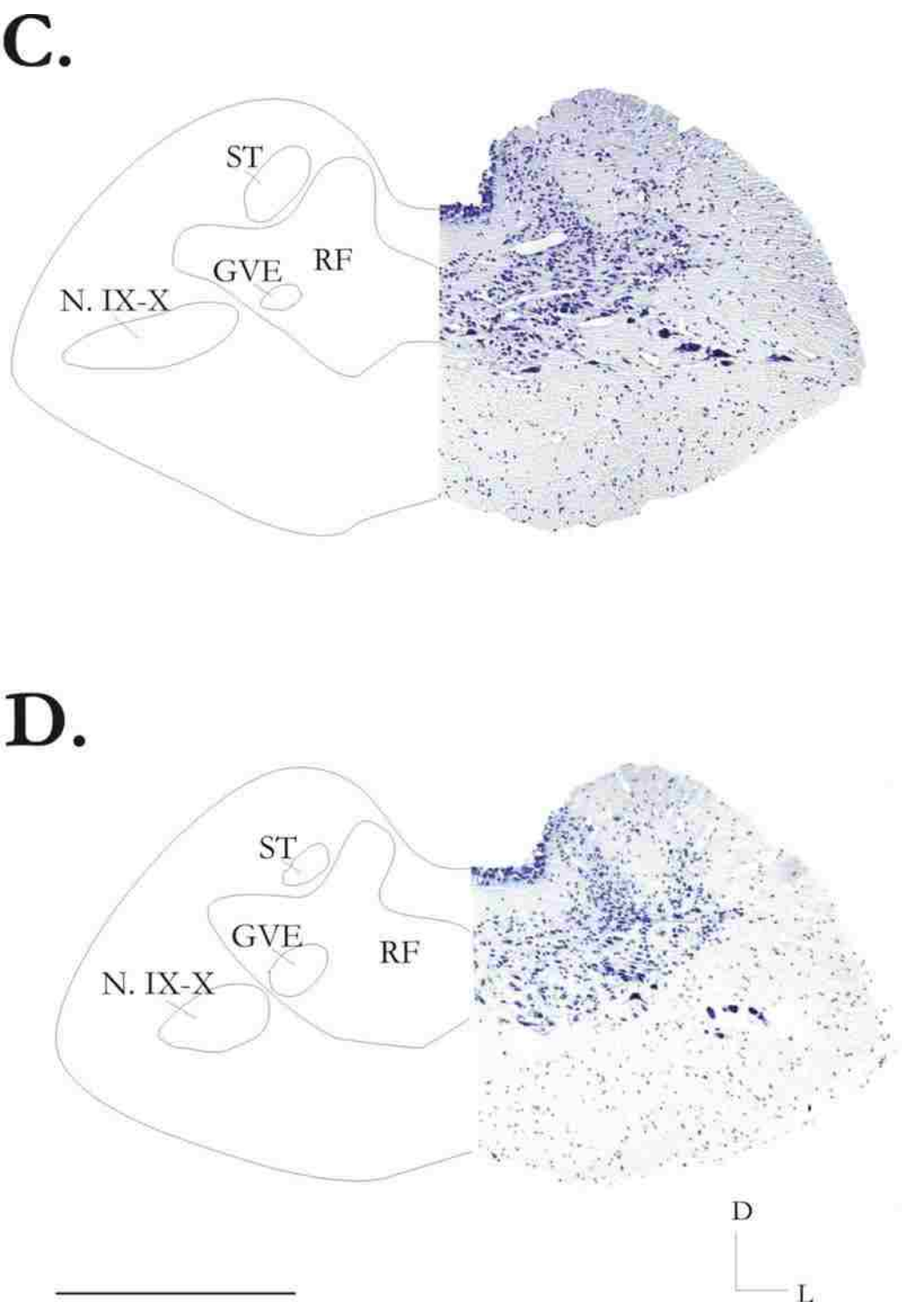


Figure 8-2

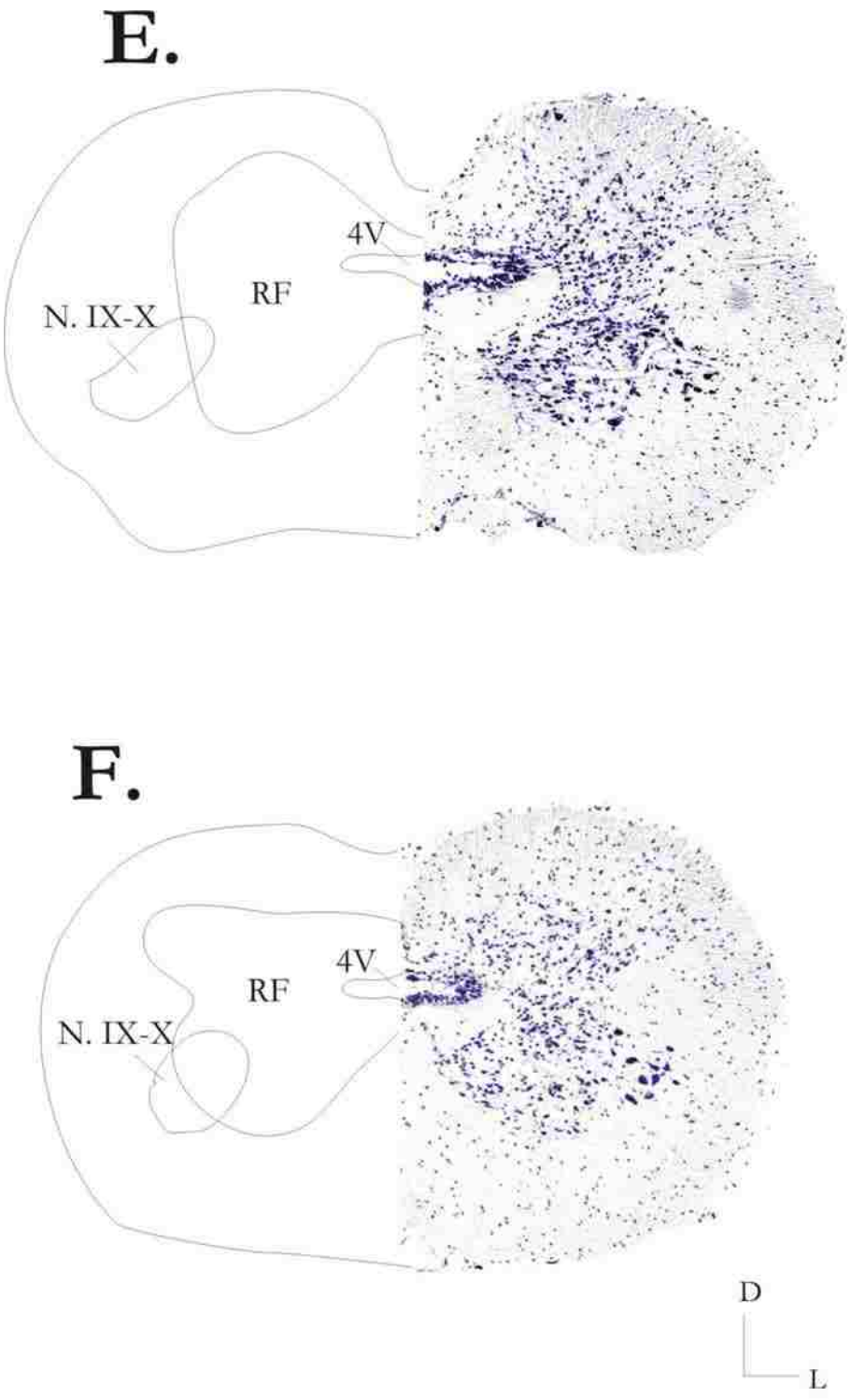


Figure 8-3

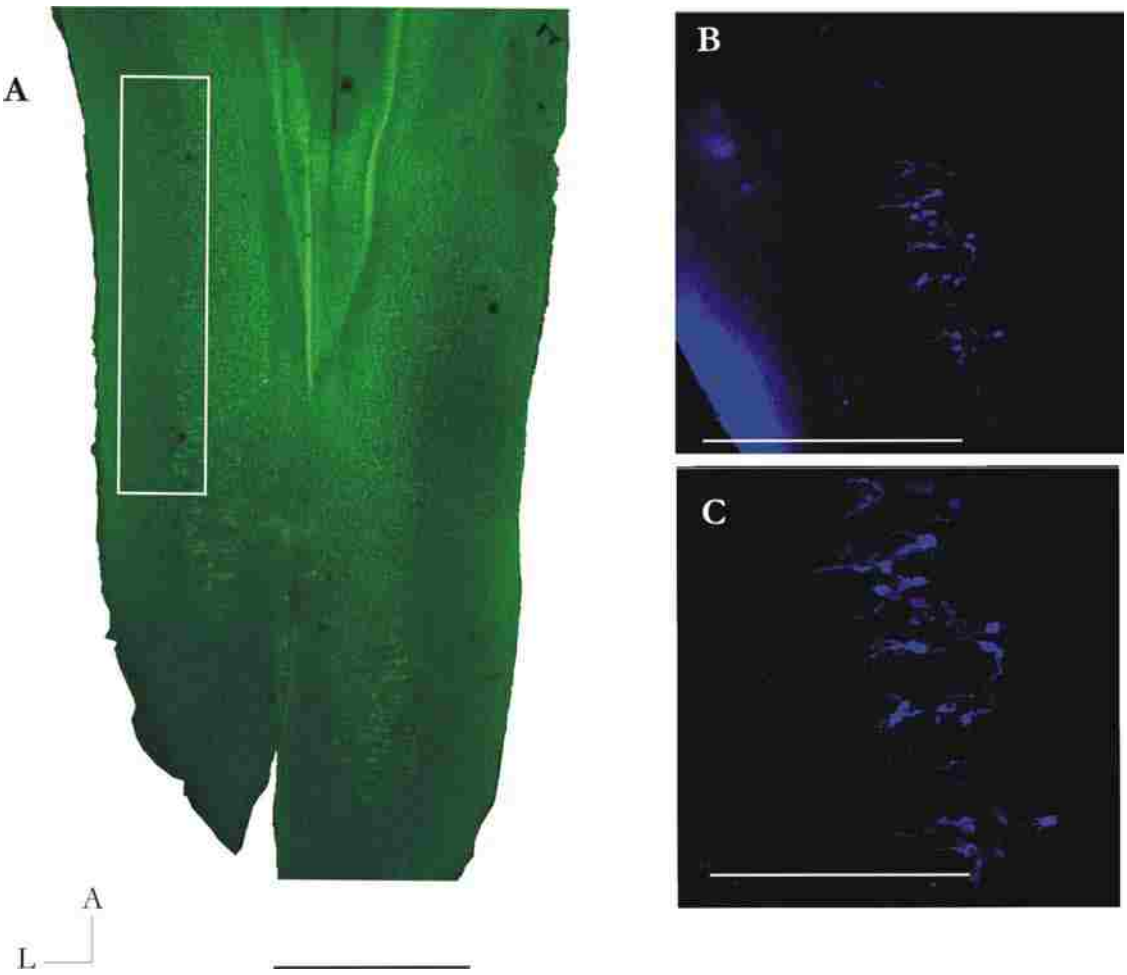


Figure 9

Figure 9. Representative micrographs of stains used to locate n. IX-X. A. Confocal micrograph (4x) of fluorescent Nissl stained horizontal section through the hindbrain area containing n. IX-X. White line highlights n. IX-X. Black scale bar represents 1 cm. B. Retrograde lipophilic diD was applied to the severed 4th root of N. IX-X of a fixed male brain (4x). Micrographs were taken after 6 month's incubation diD labeled n. IX-X somata. White line represents 1.0 cm. C. Close-up (20x) of diD labeled n. IX-X neurons. White scale bar represents 0.5 cm.

CHAPTER 5
PBDE-209 AFFECTS *X. laevis* MALE n. IX-X NEURON SIZE AND POPULATION REGARDLESS OF CRITICAL PERIOD

The vocal system of male *Xenopus laevis* endures vast changes in both anatomy and physiology during the six-month developmental critical period that follows metamorphosis. Androgens orchestrate these developmental changes that include the addition of muscle fibers to the larynx as well as efferent connections from the vocal motor nucleus (n. IX-X) to the laryngeal dilator muscles (Kelley, 1986). Androgens accumulated in n. IX-X during metamorphic climax provide a protective effect from the programmed apoptosis of n. IX-X neurons during the developmental critical period (Sassoon et al., 1986). By the time the male *X. laevis* reaches the end of the developmental critical period, the male vocal system, including n. IX-X, show measurable sex differences. Male frogs have larger and more numerous n. IX-X neurons that help to mediate the adult male frogs' more elaborate male advertisement calls (Moore et al., 2005).

The male *X. laevis* vocal system provides an ideal model for testing critical period-specific actions of a putative neurotoxin and endocrine disruptor. The critical period signals a time when brain formation relies on precise biochemical events and very specific levels of hormones for proper structural and physiological development. Once the male *X. laevis* reaches adulthood, n. IX-X is relatively resistant to morphological change, while alterations made during the developmental critical period may be

permanent and irreversible. The adult vocal system is just as reliant on specific events and androgens for vocalization. Changes in function, however, are usually more plastic than changes in adult morphology.

The present study utilized critical period and post-critical period male *X. laevis* to test PBDE-209's putative teratogenic and neurotoxic effects on the vocal system. For the first study, PBDE-209 was administered to *X. laevis* beginning at the early larval period through sexual maturity. Animals remaining at the experiment's end were surveyed for changes in growth parameters as well as for skewed sex ratio. Male *X. laevis* brains were then dissected and sectioned for analysis of n. IX-X neuron count and size. In a separate experiment, adult male *X. laevis* were treated with PBDE-209 for a six-week period after which n. IX-X neurons were measured and quantified. Results for the juvenile critical period experiment will be presented first followed by the results for PBDE-209 treatment in adult frogs.

The developmental critical period is crucial in establishing the anatomy necessary for determining an organism's reproductive capabilities. Exposure to agents that disrupt the precise mixture and action of hormones and cellular organization processes can permanently damage reproductive tissues especially if they are being organized during a critical hormone-sensitive time. The vocal motor system of *Xenopus laevis* is one such group of tissues that depends heavily on the specific actions of hormones and biochemical processes to correctly build essential organs for mating and reproduction. Thus exposure to the purported anti-androgen, PBDE-209, should directly affect androgen-dependent tissues like n. IX-X.

The data collected show that PBDE-209 did not affect the growth of the treated frogs at any concentration. There was no difference among or between PBDE-treated frogs in both body length (Figure 10, p =0.1465) and weight (Figure 11, p = 0.2812). Moreover, sex-specific differences in length and weight were, for the most part, maintained with females usually bigger and heavier than males. In the low dose (0.1 ppb PBDE-209) group males were slightly longer than females, while in the middle dose (1.0 ppb PBDE-209) group males and females were relatively similar in size. Sex differences in size and weight are probably more firmly established in adulthood after the frog has access to more food and a larger environment and are most likely unaffected by PBDE-209. All frogs metamorphosed within seven days of one another, so there was no affect of PBDE-209 on metamorphic timing. Among survivors, there appeared to be no difference in the ratio of males to females among the treatment groups (Figure 12, p = 0.295). A chi square test determined there was no difference among treatment groups.

PBDE-209 did not influence growth parameters or sex ratio, its effects, however, seemed more targeted toward the androgen-sensitive vocal tissues. The motor nucleus n. IX-X was most affected by PBDE-209 treatment. Before quantifying the number of cells per vocal motor nucleus, the precise location of n. IX-X was determined. Figure 13 shows a gross dissection of the brain of a newly sexually mature male frog. Even when newly sexually mature, n. IX-X fibers are easily visible as they exit the brain via root 4 of the glossopharyngeal-vagal cranial nerve (N. IX-X complex), a four-root complex for which the fourth and most caudal root consists solely of motor axons innervating the laryngeal dilator muscles. A line drawing of the *X. laevis* brain (Figure 14A) illustrates the approximate location of n. IX-X in the caudal medulla of the hindbrain. The nucleus

lies 400-500 μm below the dorsal surface of the 1000-1200 hindbrain and runs caudally from the N. IX-X insertion to just caudal of the obex and anterior to the first spinal roots.

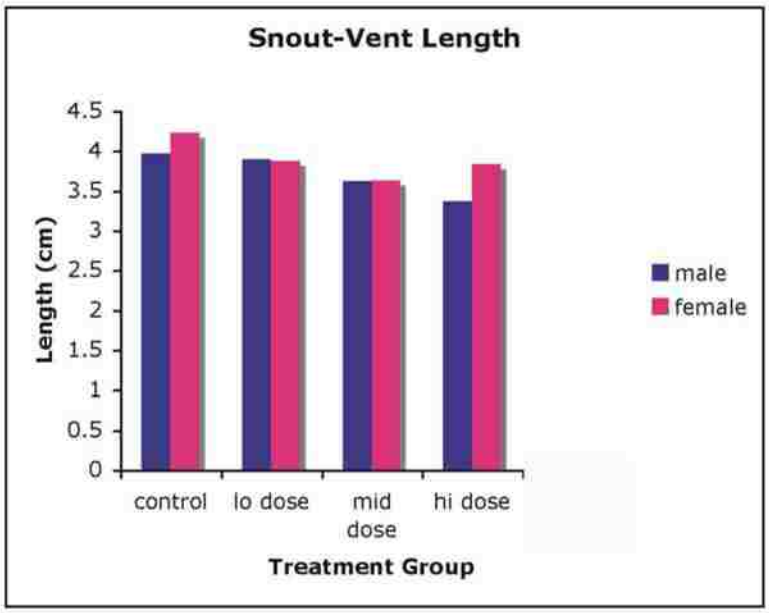


Figure 10

Figure 10. Embryos exposed to PBDE-209 for the duration of the larval period and six months after metamorphosis showed no significant effects of treatment on growth parameters. PBDE-209 had no effect on post-treatment weight. There was no significant difference in snout vent length (SVL) among treatment groups when compared using a one-way ANOVA ($p = 0.147$).

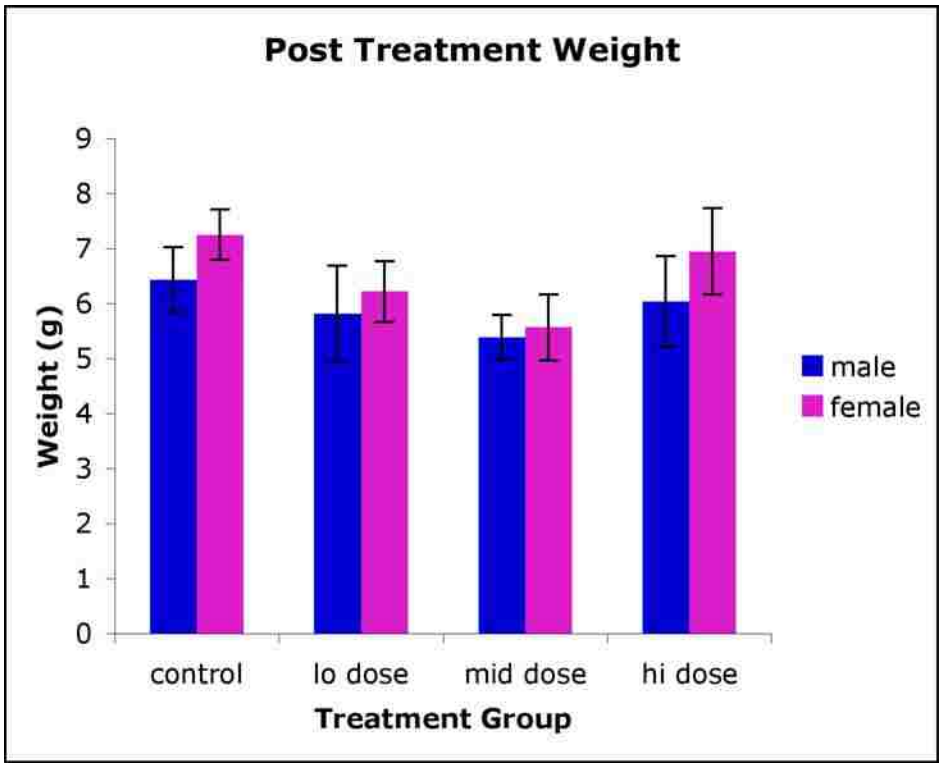


Figure 11

Figure 11. Growth parameters like SVL (see Figure 9) and weight were not affected by PBDE treatment. A one-way ANOVA comparing frog weights showed that there was no difference among PBDE-209 treated and control frogs ($p = 0.147$).

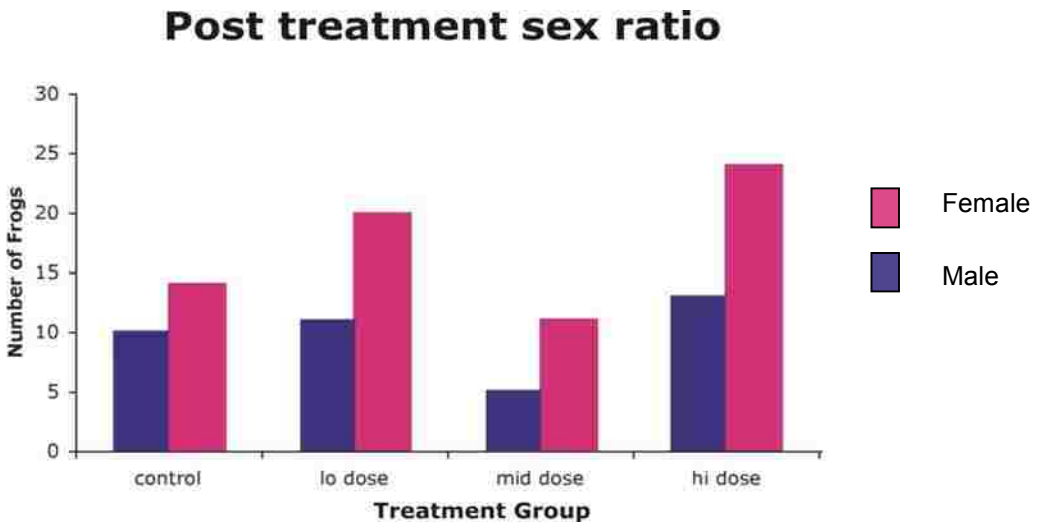


Figure 12

Figure 12. One capability of a strong anti-androgen would be to skew sex ratio to be female biased. PBDE-209 has had purported anti-androgenic effects on developing male mice but not on sex ratio (Lilienthal et al., 2006). Chi square analysis showed that PBDE-209 at any concentration did not have significant effects on sex ratio ($p = 0.295$).

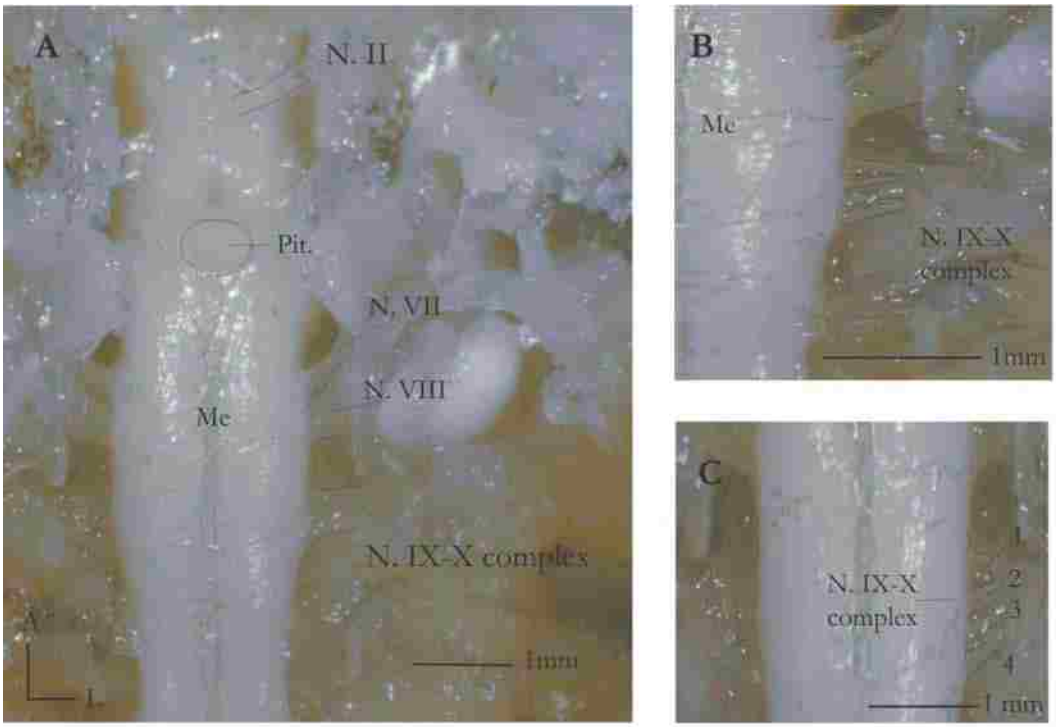


Figure 13

Figure 13. A. Gross dissection showing the ventral surface of a newly sexually mature male *X. laevis*. B. Close-up of the area of cranial nerve N. IX-X complex insertion. C. Detail of N. IX-X illustrating the 4-root complex, the 4th root of which is the motor nerve to the larynx. Abbeviations: N. II = optic nerve, N. VII = facial nerve, N. VIII = auditory nerve, N. IX-X complex = glossopharyngeal-vagal nerve complex, Me = medulla, n. IX-X = vocal motor nucleus, Pit. = pituitary, 1-4 = roots of N. IX-X. Line represents 1 mm.

Figure 14

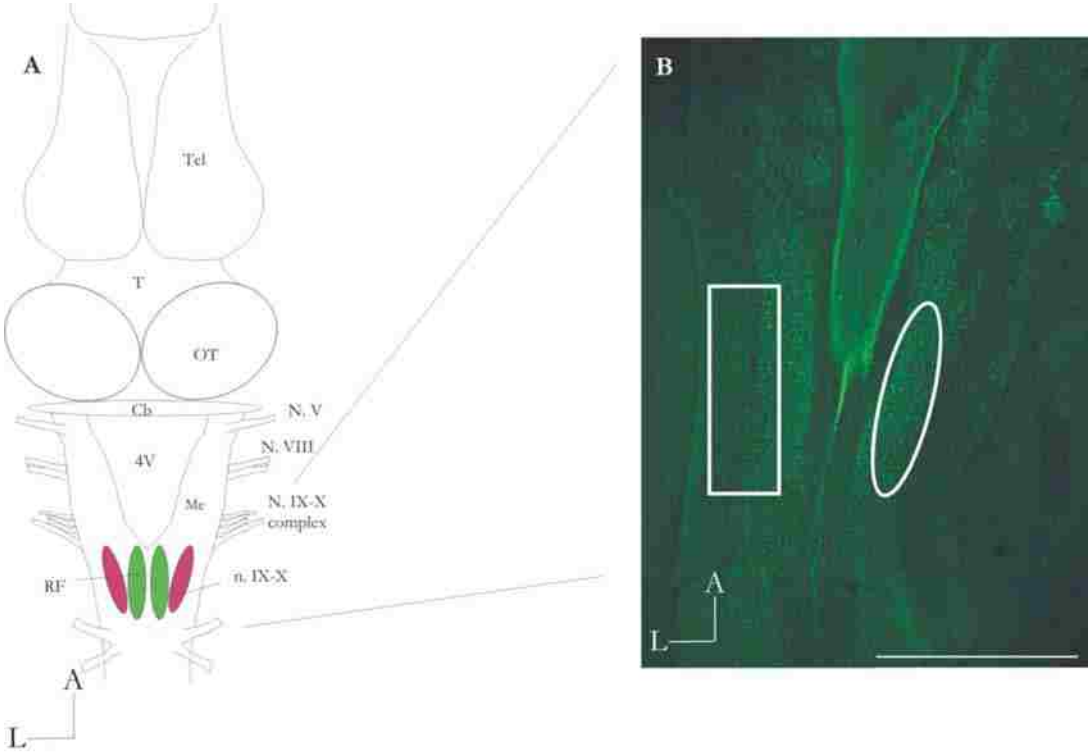


Figure 14. A. Line representation of the vocal nuclei in the hindbrain of *X. laevis.* Cb = cerebellum, Me = medulla, N. V = trigeminal nerve, N. VII = facial nerve, n. IX-X = vocal motor nucleus, N. IX-X complex = glossopharyngeal-vagal nerve complex, OT = optic tectum, RF = reticular formation, T = thalamus, Tel = telencephalon, 4V = fourth ventricle. B. Conofocal micrograph (4x) of a fluorescent Nissl stained section through the hindbrain of male *X. laevis.* White oval encircles motor pattern generator, RF, and white rectangle surrounds n. IX-X. Line = 1cm.

The central motor patterning unit, the reticular formation, runs medial to the n. IX-X and provides motor pattern generation for n. IX-X mediated mate vocalizations.

The location of n. IX-X was reliably identified in each brain analysis, thus accurate cell counts could be made at a depth of 400-500 μm from the dorsal surface of the hindbrain (Figure 13B). Analyses were made from 100 μm horizontal serial sections through the hindbrain area containing n. IX-X. The entire nucleus was 200-300 μm thick in the horizontal plane, so the neurons in the 100 μm section for which n. IX-X was most populous were counted. Ranges of n.IX-X populations show that though counts within treatments were relatively similar, there were differences within treatment groups. Average counts of n. IX-X neurons varied significantly among treatments (Figure 15, $p = 0.003$). Neuron number did not differ among control and low dose frogs. Control frogs had more neurons than middle ($p = 0.019$) or high dose ($p = 0.002$) frogs. Though they did not differ significantly between control countss, low dose frogs also had more n. IX-X neurons than middle (0.020) or high dose (0.005) frogs. Neuron number was not significantly different among middle and high dose frogs. Surprisingly the middle dose frogs had fewer n. IX-X neurons than the other treatment groups.

The cell bodies of n. IX-X neurons also show the effects of PBDE-209 treatment (Figures 16-17). Careful inspection of the cell bodies reveals both quantitative and qualitative morphological differences. Recall that the middle dose frogs had the fewest n. IX-X neurons, but also some cells had the most peculiar morphology post-treatment (Figure 17A), appearing nested within tissue, and measuring highly significantly different in both length and width than control ($p < 0.001$) and low dose ($p < 0.001$) frogs but not statistically different in length ($p = 0.139$) and width ($p = 0.118$) compared to high dose

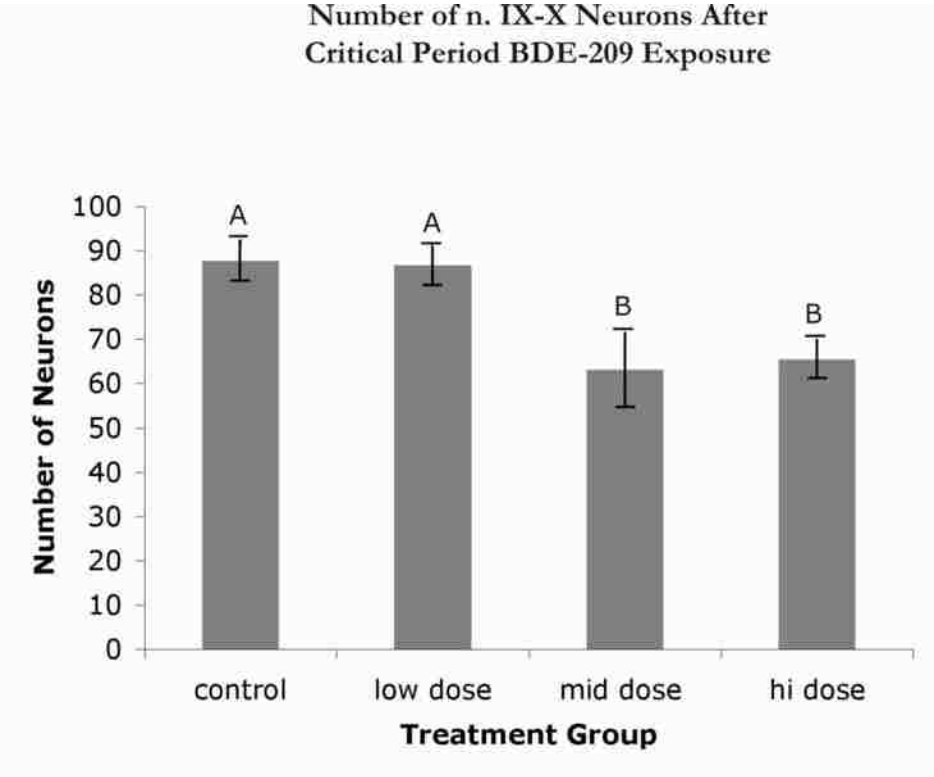


Figure 15

Figure 15. One-way ANOVA compared n. IX-X neuron counts among newly sexually mature PBDE-209 treated male frogs. There was a significant difference in cell count among treated frogs ($p = 0.003$). Newman-Keuls multiple comparisons post-hoc tests revealed that there was no difference in neuron count between control and low dose frogs ($F = 25.46$, $df = 4$, $p = 0.000$, $n = 47$). There was also no difference between counts in middle dose and high dose frogs. Middle and high dose frogs, however, had significantly fewer n. IX-X neuron cell bodies than control and low dose frogs.

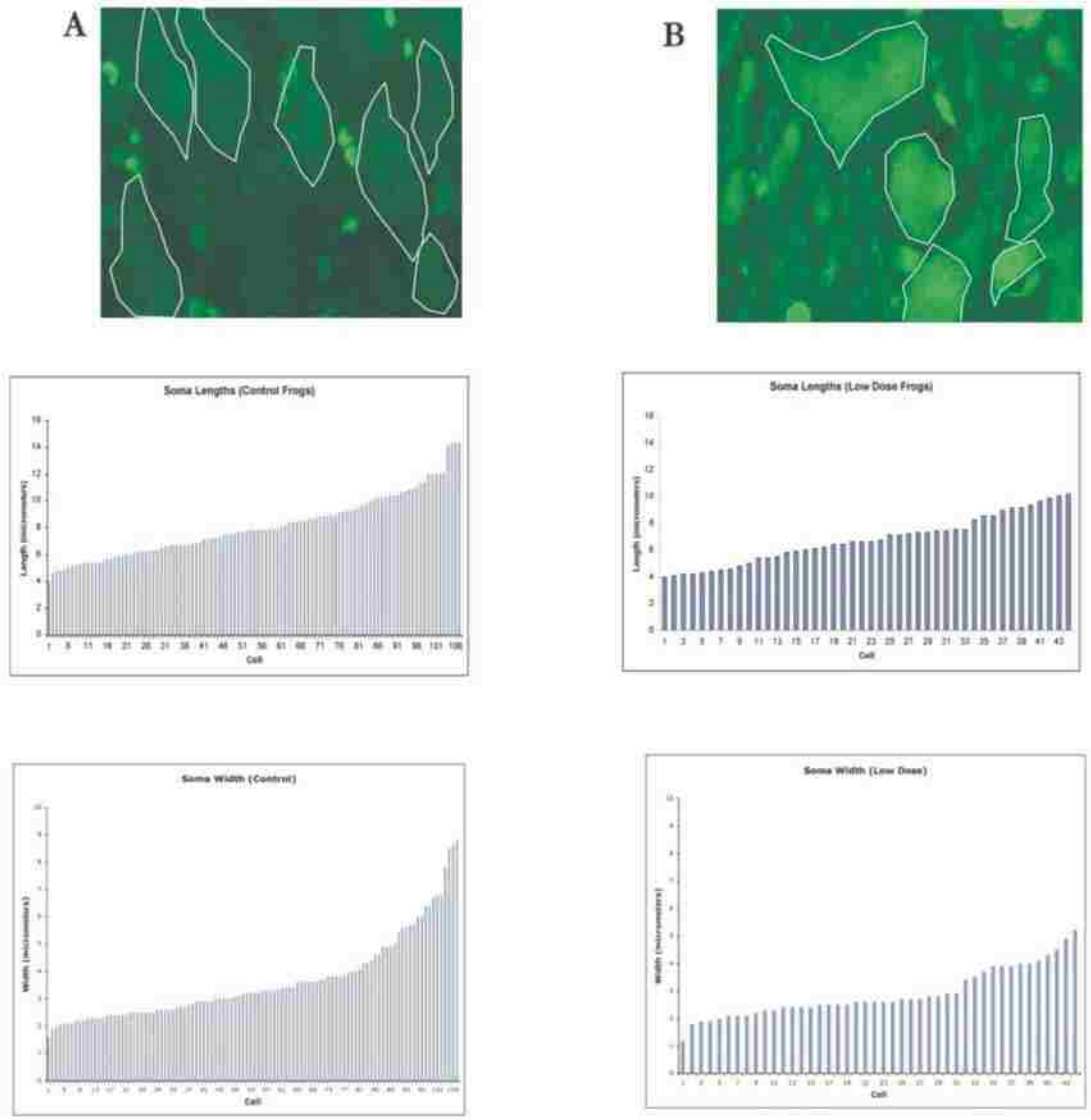


Figure 16

Figure 16. Examples of n. IX-X somata in A. control, B. low dose, and C. middle dose frogs. Histograms in the second row describe ranges in soma length and width in the third row. As treatment with PBDE-209 increased, cell counts and cell size decreased. Note the peculiar appearance of n. IX-X somata in the middle dose group C. Neurons measured in the middle dose group were significantly smaller than control ($p < 0.001$) and low dose ($p < 0.001$) groups.

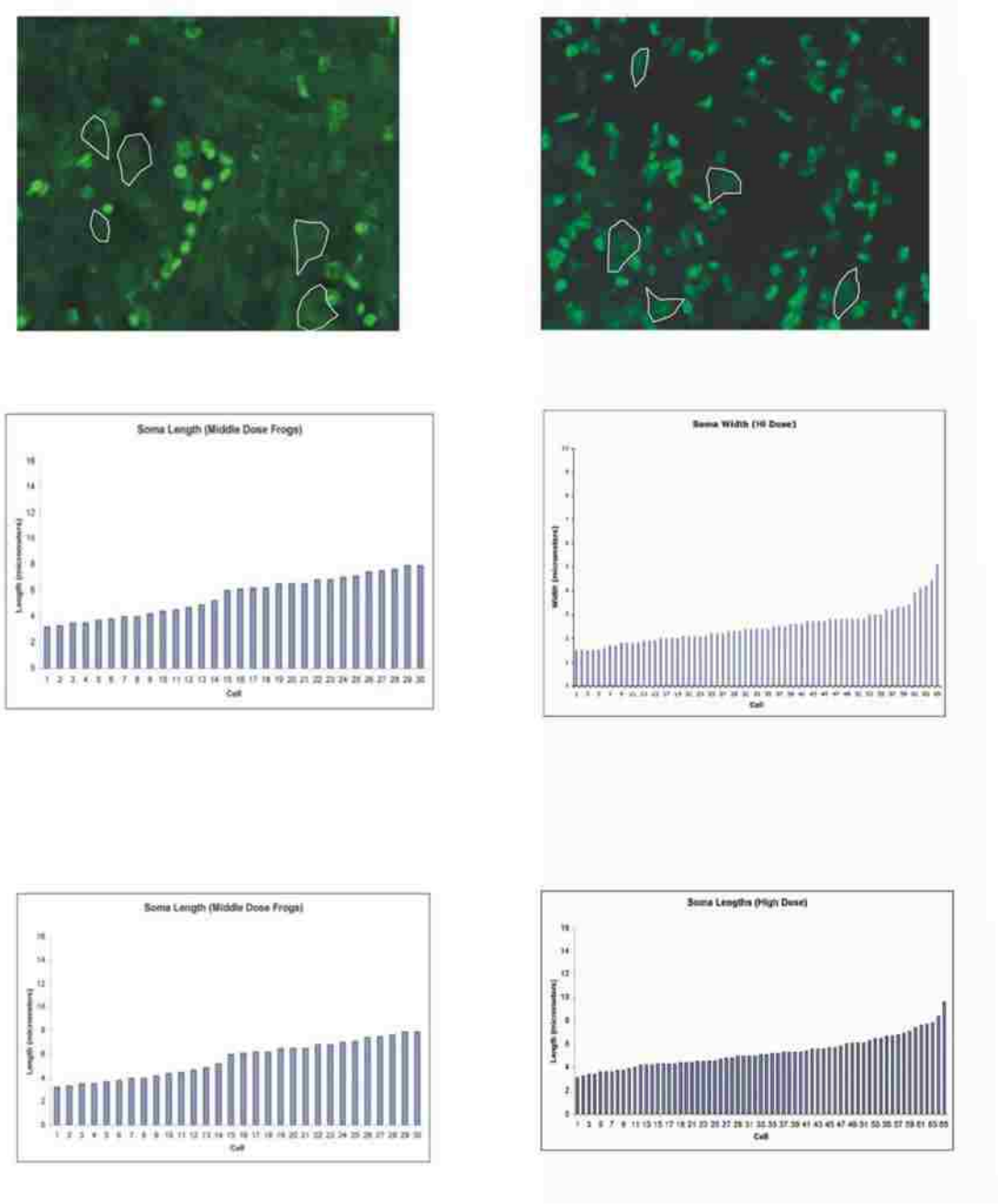


Figure 17

Figure 17. Confocal micrographs (20x) show examples of n. IX-X neurons after middle dose and high dose PBDE-209 treatments during the developmental critical period. . Beneath each micrograph are histograms describing the ranges of n. IX-X somatal length and width. A. Middle dose frogs had the fewest neurons of all frogs after treatment but not the smallest. B. High dose frogs had significantly fewer neurons after treatment compared to control and middle dose frogs, and their n. IX-X neurons were also smaller than those of the other frogs.

groups. Somata lengths and widths were measured in each treatment group and ranges for each parameter were plotted (Figures 18-19). Though significantly different, lenghts and widths of somata in the middle dose group fell within the range of the control and low dose somata. The n. IX-X cell bodies of high dose frogs were significantly smaller than control and low dose frogs The developmental critical period signals a time of orchestrated change in male *X. laevis*. Goverened by the actions of hormones and cell growth and death processes, the sexually different vocal system is formed. Once the tissues underlying mate calling are established during the critical period, it is very difficult to alter tissue anatomy. While the frogs are developing, however, differences in n. IX-X neuron number and size were recorded after treatment with the purported neurotoxin and endocrine disruptor, PBDE-209. Because adult frogs have already established their n. IX-X anatomy, the present study predicted there would be no effect of PBDE-209 on adult male n. IX-X neuron population or size.

Adult male *X. laevis* were exposed to environmentally relevant levels of PBDE-209 for six weeks (Table 2). For PBDE-209 treated adult male frogs, changes in the laryngeal motor nucleus, (n. IX-X) were quantified as with the critical period-treated frogs: 1) by counting the number of neurons in n. IX-X, and 2) by measuring n. IX-X neuron size. Neuron counts indicate that as PBDE-209 exposure increased, neuron number decreased. In the control group, n. IX-X neuron counts ranged from 70-150 cells, 90-126 cells for low dose frogs, 94-110 in the middle dose, and 55-112 in high dose frogs. Statistical analyses indicate that neuron counts of treated frogs vary significantly from one another (Figure 20). High dose frogs had fewer n. IX-X neurons than control ($p = 0.003$), low dose ($p = 0.013$), and middle dose ($p = 0.012$) frogs.

Average n. IX-X Soma Length After PBDE-209 Treatment During the Developmental Critical Period

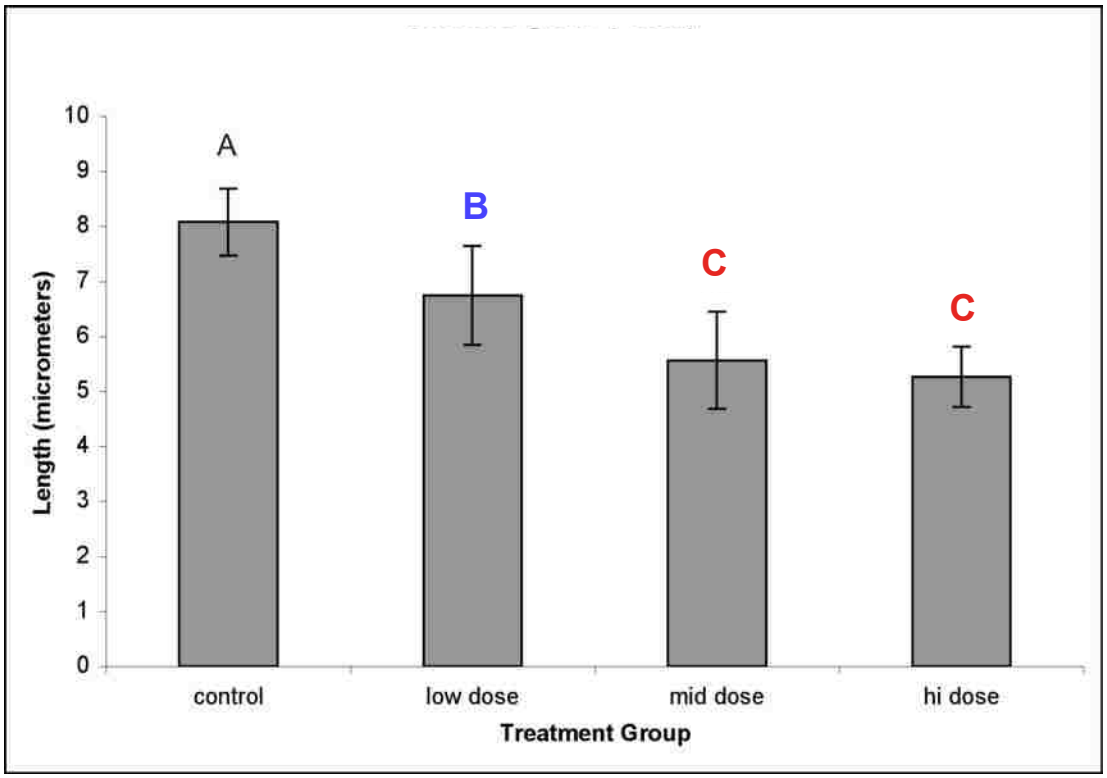


Figure 18

Figure 18. Lengths of n. IX-X neurons were compared among newly sexually mature PBDE-209 treated male frogs. A one-way ANOVA indicated that there were significant differences in the average length of n. IX-X neurons after PBDE-209 treatment ($F = 61.29$, $df = 3$, $n = 352$, $p = 0.001$). Student Neuman-Keuls multiple comparisons post hoc test revealed significant differences between control and low, middle, and high dose frogs ($p < 0.001$ for all control group comparisons), significant differences between low and middle dose groups ($p = 0.03$), but no significant difference between middle and high dose groups.

Average n. IX-X Soma Width After PBDE-209 Treatment During the Developmental Critical Period

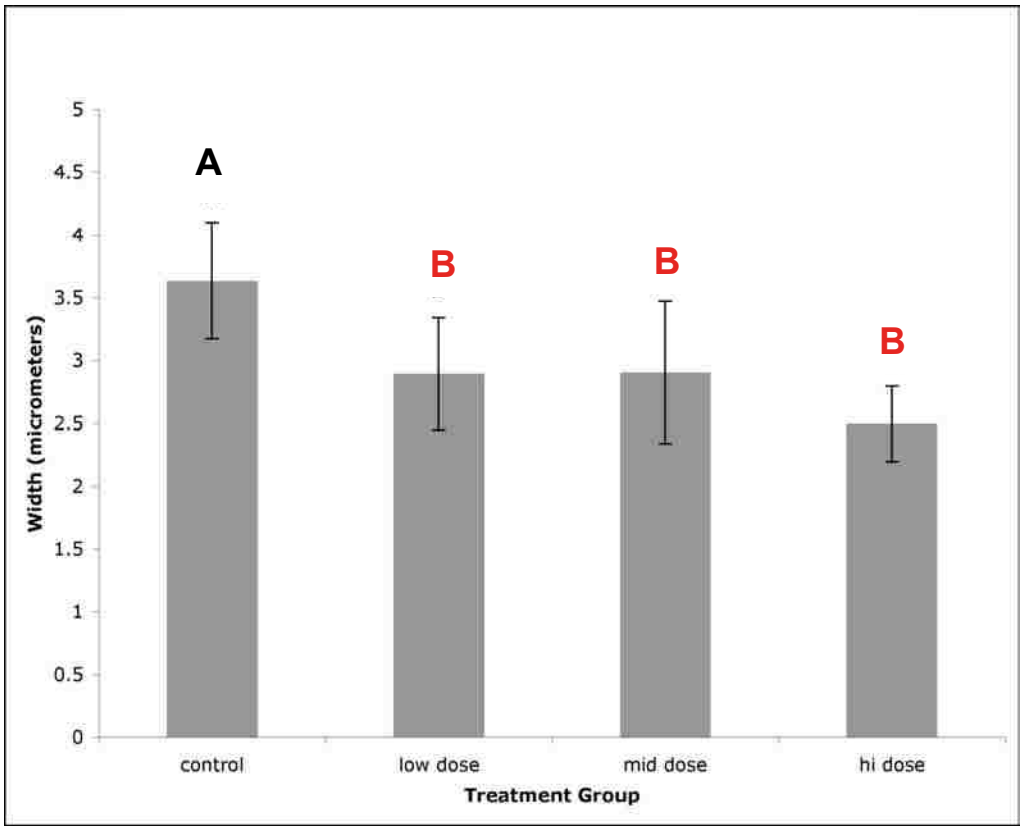


Figure 19

Figure 19. Widths of n. IX-X neurons were compared among PBDE-209 treated frogs. A one-way ANOVA indicated that there were significant differences in the average length of n. IX-X neurons after PBDE-209 treatment ($F = 31.06$, $df = 3$, $n = 352$, $p < 0.001$). Student Neuman-Keuls multiple comparisons post hoc test revealed significant differences between control and low, middle and high dose frogs ($p < 0.001$ for all both comparisons). There was not a significant difference in soma width between low and middle and high dose groups.

Average Number of n. IX-X Neurons in Adult Male *X. laevis* After PBDE-209 Treatment

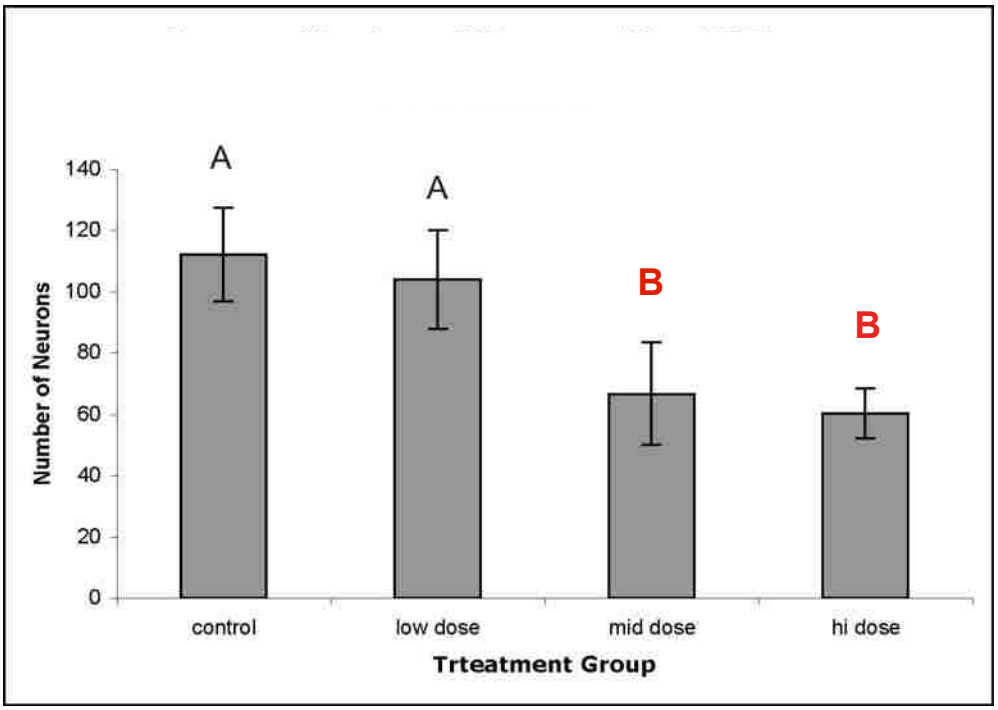


Figure 20

Figure 20. After six-weeks treatment PBDE-209 treatment, there were significant differences in neuron number. One-way ANOVA showed statistically significant differences in n. IX-X neuron number among treatment groups ($F = 25.46$, $df = 4$, $n = 25$, $p < 0.0001$). Student Newman-Keuls multiple comparisons tests revealed the sources of variation. There were no differences in neuron count between control and low dose frogs. Comparisons of cell counts between middle and high dose groups also did not differ significantly from one another. Differences, however, were significant between control and low dose frogs and middle and high dose frogs.

Counts of n. IX-X neurons in control, low dose, and middle dose frogs did not differ significantly from one another.

PBDE-209 treated frogs varied not only in n. IX-X neuron number, but also in the size of the neurons. As PBDE-209 concentration increased, the length and width of n. IX-X neurons decreased. Though some neurons from high dose frogs fell within the control ranges for soma widths, the high dose neurons were significantly smaller than the control and low dose frogs ($p < 0.001$ for both comparisons), but not middle dose frogs ($p < 0.118$). Positive control frogs also had strongly significantly smaller n. IX-X neurons than all other groups even the high dose frogs ($p < 0.001$). Control frogs possessed significantly longer somata than all other treatment groups, and as PBDE-209 concentration increased, neurons were highly significantly shorter than the groups receiving less PBDE-209 except middle and high dose frogs did not differ significantly in length from one another ($p = 0.139$). Examples of n. IX-X somata size variations are found in Figures 21 and 22, where differences in cell size and appearance are indicative of PBDE-209's effects.

As PBDE-209 treatment concentration increased, n. IX-X neuron populations decreased and were smaller in both length (Figure 23) and width (Figure 24). Both length and width varied significantly among treatments ($p < 0.001$). These changes are contrary to previous studies (Hannigan and Kelley 1986, 1983; Simpson et al. 1986) that found that organization of the androgen governed vocal system is usually fixed, and also contrary to our hypothesis that PBDE-209 mediated changes are critical period restricted.

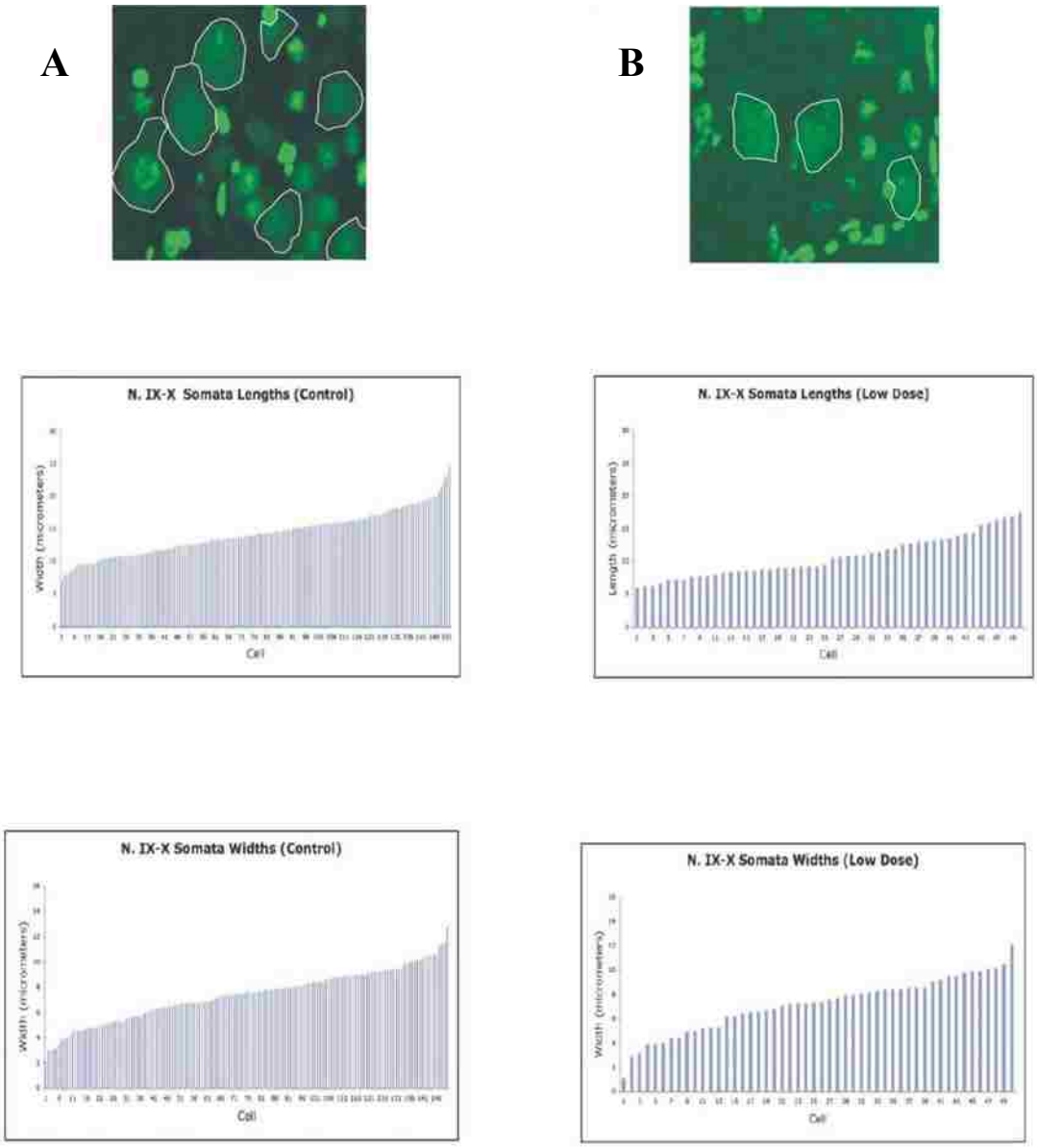


Figure 21

Figure 21. The average adult male *X. laevis* possesses larger and more numerous n. IX-X neurons than females. Representative confocal micrographs of A.) Control frog n. IX-X somata and B.) Low dose frog n. IX-X somata show typical, large (15 μm length, 12 μm width) cells. Measures of n. IX-X neuron length and width did not differ significantly between control and low dose frogs.

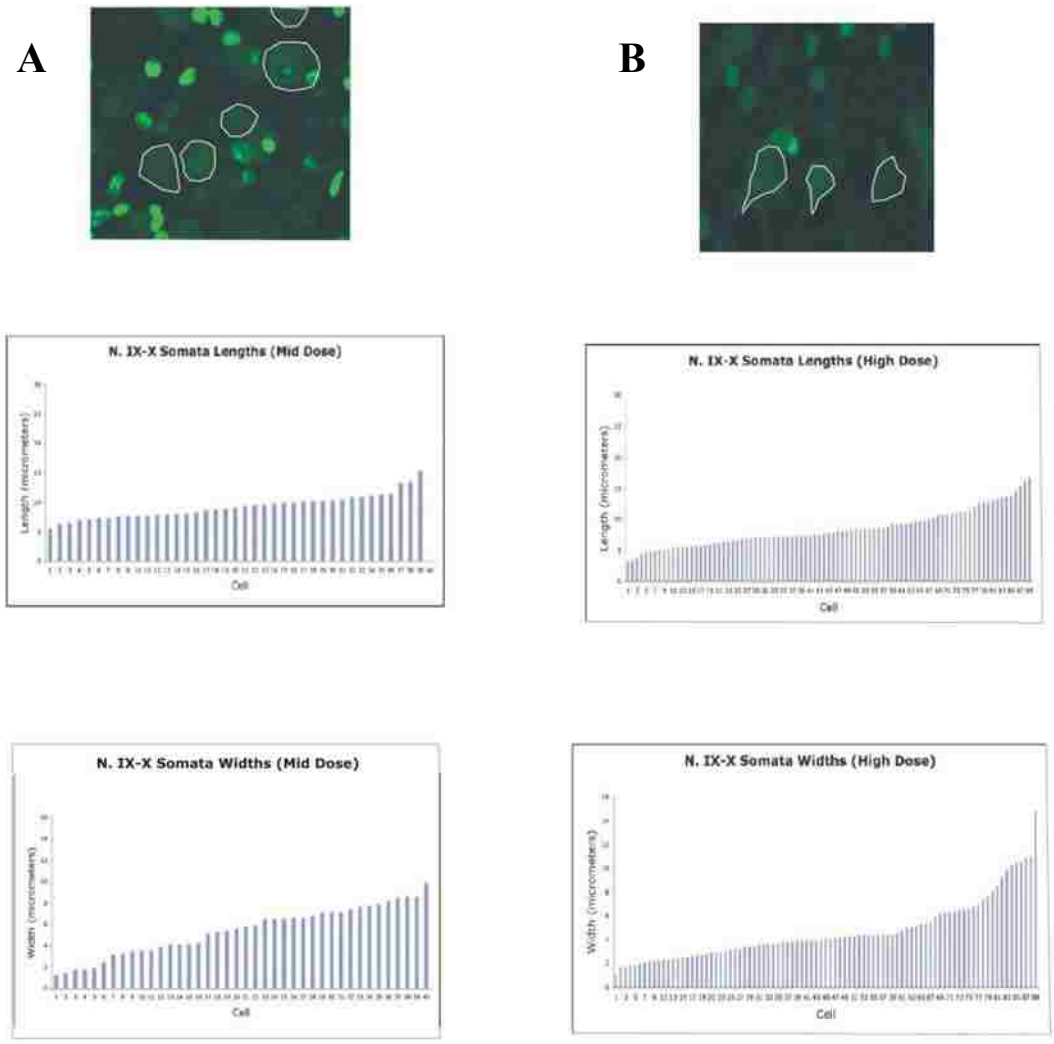


Figure 22

Figure 22. Representative micrographs of n. IX-X somata from: A. Middle dose frogs and B. High dose frogs. Confocal micrographs and measures of soma length and width indicate that as PBDE-209 treatment increased, cell size decreased. Somata were shortest in high dose groups. Measures of soma width did not differ between middle and high dose groups but were significantly smaller than control and low dose somata.

Average Length of n. IX-X Neurons in Adult Male *X. laevis* After PBDE-209 Treatment

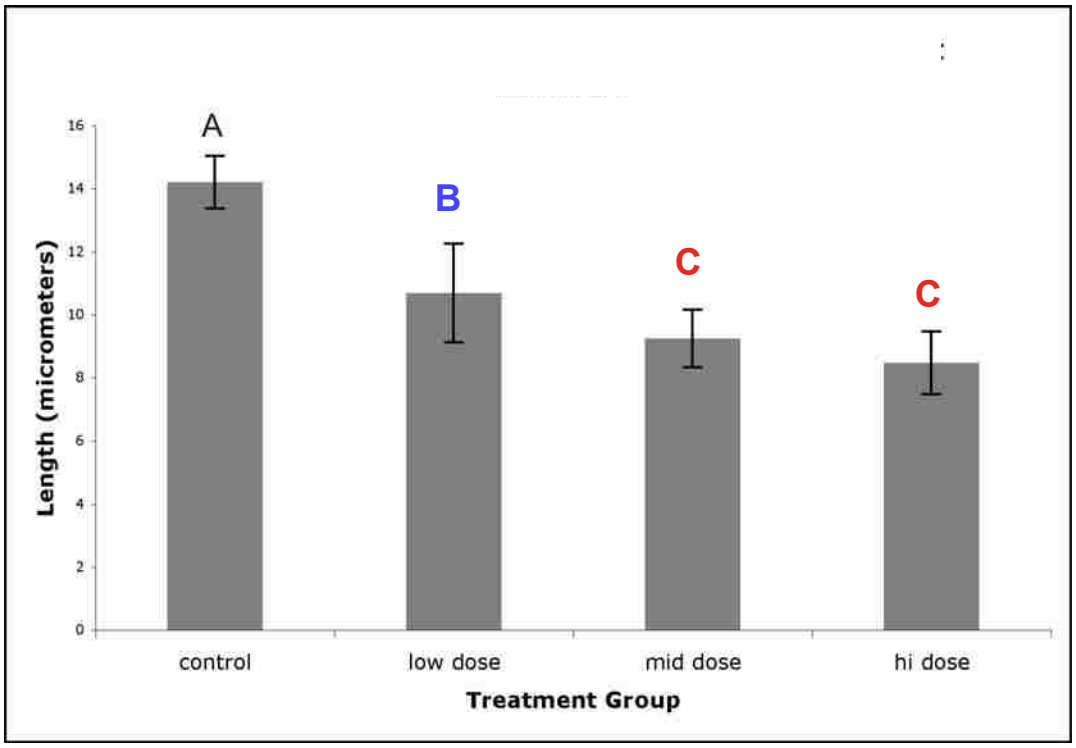


Figure 23

Figure 23. As PBDE-209 concentration increased, cell size decreased. High dose neurons were significantly smaller than the control and low dose frogs ($p < 0.001$ for both comparisons), but not middle dose frogs ($p < 0.118$). Control frogs possessed significantly longer somata than all other treatment groups, and as PBDE-209 concentration increased, neurons were highly significantly shorter than the groups receiving less PBDE-209 except middle and high dose frogs did not differ significantly in length from one another ($p = 0.139$).

Average Adult Male n. IX-X Soma Width
After PBDE-209 Treatment

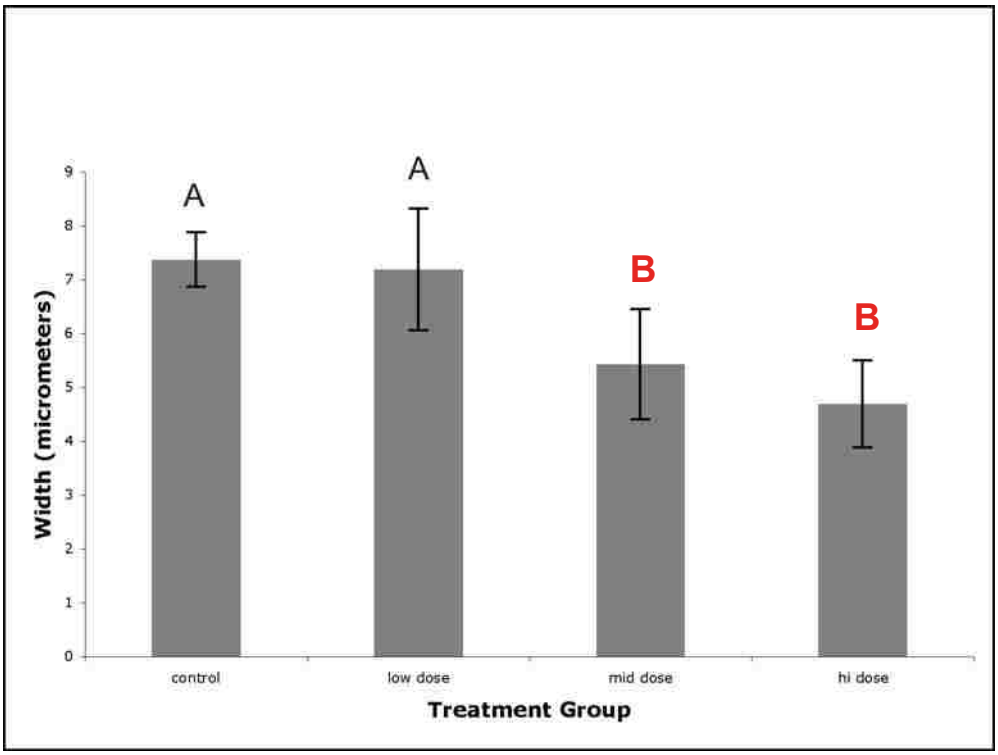


Figure 24

Figure 24. Increasing concentrations of PBDE-209 had an effect of decreasing n. IX-X neuron size in adult male *X. laevis*. There was no difference in soma width between middle and high dose frogs ($p < 0.118$). High dose and middle dose neurons, though, were significantly smaller than the control and low dose frogs ($p < 0.001$ for both comparisons).

Discussion

Reproductive organs are formed during developmental critical periods when the influences of hormones and the actions of precisely orchestrated cellular processes shape these organs and govern how they will function during adulthood. Hormone-mimicking chemicals, or endocrine disruptors and teratogens may alter development, thus exposure during critical periods will likely affect the proper organization of critical period sensitive tissues. The vocal system of the African Clawed frog, *Xenopus laevis*, is an organ system that is formed during a developmental critical period that lasts for a six month period immediately after metamorphosis when developing male frogs are highly sensitive to androgens. Shortly before metamorphic climax, cells in the vocal motor nucleus in the hindbrain begin to concentrate androgens, and production of androgen receptors increases in the peripheral vocal tissues (Kelley, 1986; Sassoon et al., 1987). Androgen levels in males increase dramatically during the six-month critical period, while levels in females diminish, as androgens seem to protect n. IX-X cells from programmed death (Kelley et al., 1987; Wetzel et al., 1986; Kay et al., 1999). Sex differences in n. IX-X number are not noticeable until the end of the critical period (Gorlick and Kelley, 1987). By the time the male frog reaches sexual maturity at the close of the developmental critical period, it has 40% more n. IX-X neurons than the female. The neurons making up n. IX-X are not only more numerous, but also larger with more complex dendritic arbors and axonal fibers to make many synaptic connections with the robust male larynx (Kelley, 1986; Wetzel et al., 1986; Kelley et al., 1988; Zornick and Kelley, 2007).

Androgen levels and developmental processes during the critical period are, therefore, essential to forming the male *X. laevis* vocal system. Androgens are so

influential, that treatment of females nearing metamorphosis with androgens greatly increased the survival of n. IX-X neurons to male levels (Kay et al., 1999). Androgen treatment is also capable of masculinizing female vocalization by changing n. IX-X soma size (Hannigan and Kelley, 1986; Potter et al., 2005) and restoring vocalization capabilities to castrated males (Watson and Kelley, 1992). Though some adult females treated with androgens had male-like vocalizations, androgen treatment had only trophic effects and did not initiate the addition of any new n. IX-X cells (Hannigan and Kelley, 1986). Because vocal organ formation in *X. laevis* is so heavily reliant on critical period developmental processes, the present study sought to explain how treatment with the putative anti-androgen and neurotoxin, PBDE-209, would affect the organization of the vocal organs.

Androgens are critical to the formation of the vocal system. Male frogs rely on vocalizations to attract mates, and though female *X. laevis* elicit vocalizations that either advertise their reproductive readiness (rapping = fast series of vocal clicks of similar small amplitude) or disinterest in an approaching male (slow ticking = very slow series of similar small amplitude) their vocal anatomy and physiology prevent the females from ever producing male-typical calls consisting rapid clicks of variable amplitude. Thus, the establishment of proper vocal organs determines a male frog's ability to attract mates and reproduce.

Treatment of frogs at environmentally relevant levels of PBDE-209 yielded deficits in n. IX-X, the hindbrain motor nucleus in charge of mitigating vocalization during mating. PBDE-209 treatment resulted in frogs with fewer n. IX-X neurons that were also statistically smaller than control or low dose treated frogs, effects that could

severely impair a frog's ability to vocalize to attract mates and reproduce. The highest concentrations of PBDE-209 often yielded the frogs with the fewest n. IX-X neurons and the smallest remaining neurons. The highest concentration of PBDE-209 used in the present study (10 ppb) is several times less than the 212 ppb concentrations found in sediment samples from North America (Oros et al., 2005).

Kay et al. (1999) showed that after castration of developing juvenile male frogs, the absence of androgens severely lessened the resultant number n. IX-X neurons. Without appropriate androgen levels, the male vocal system could never produce male-typical calls necessary for attracting mates. Though androgen's trophic effects on n. IX-X cell size may enable some female frogs to make male typical calls, increases in n. IX-X cell size allowed only 22% of experimental frogs to make male-like calls (Hannigan and Kelley, 1986). Proper n. IX-X cell size as well as cell number are critical for male-typical vocalization. The resultant deficits in n. IX-X number and size in the present study are effects that would most likely impair the frog's ability to properly mate and reproduce.

Absences in androgens during critical periods of vocal system organization will result in deficits in vocal programs. Considering the ubiquity of PBDE-209 in the environment, exposure during critical periods of organ formation are likely not only in developing frogs, but also in developing humans. Detectable levels of PBDEs are present everywhere, even in our house dust, clothing, food, and also our bodies bear a heavy PBDE burden. Juvenile humans seem to bear most of the heavy PBDE concentrations (Costa and Giordano, 2007), which may impair proper development of hormone-dependent tissues. Already, positive correlations have been made between PBDE levels

in the breast milk of nursing mothers and the incidence of testis malformation. Only one of the lowest PBDE levels measured in human breast milk fell with in the relatively conservative range of PBDE concentration used in the present experiment.

Developmental constraints usually set in stone reproductive anatomy. Central dogma maintains that the critical period that defines the development of the vocal tissues and gonads gives way to permanent, unchangeable organs. In the present study, however, treatment with PBDE-209 changed the hormonally organized n. IX-X by decreasing both the number and size of its neurons. How then, can these obvious anatomical changes in adult *Xenopus* be explained? Thorough studies of the *Xenopus laevis* N. IX-X development (Wetzel et al., 1985, Kelley, 1997, and Kelley, 2002) indicate that though the presence of testicular androgens is essential to reproductive organ development, the increase in androgen receptors during the critical period seems to ensure proper development of reproductive structures, especially the vocal organs. Thus there are several routes by which hormone-mimics may exact change.

Kelley (1986) explains that the increase in androgen sensitivity during the early critical period first establishes the male's robust peripheral vocal structures. Male-typical anatomy of the vocal structures in the central nervous system soon follows. Though the vocal nuclei in the male brain begin concentrating androgens before the developmental critical period, by PM6, both central and peripheral structures are sexually different (Hannigan and Kelley, 1986; 1983; Simpson et al., 1986). Endocrine disruption or exposure to teratogens may ruin the development of the larynx or brain structures involved in mate calling.

Maintenance of these tissues and the development and growth that comes with their use during mating also depends not only on the precisely developed anatomy and physiology of vocal organs, but also on testicular androgens being released in precise amounts. Mating events necessitate the release and binding of androgens for calling and amplexus. In order for male *X. laevis* to produce the appropriate advertisement calls, vocal motor neurons must be the appropriate number and size. The larynx, song circuit, n. IX-X, and laryngeal motor nerve are all sensitive to circulating androgens during these critical periods. Thus, if an endocrine disruptor or neurotoxin prevents androgens from functioning at these late critical periods, a male frog may be unable to call and mate.

Disruption of androgen levels or neurotoxic effects by PBDE-209 are plausible explanations for the decrease in neuron number and size in adult male *X. laevis.* The seasonal or mating event-specific sex differences in the brain that accompany mate calling are due to changes in circulating hormone levels (Wetzel et al., 1985). These transient or activational androgenic effects are marked by increases in vocal motor neuron size, as well as growth of afferent song nuclei (Wetzel et al., 1985). In white-crowned sparrows, for instance, there are pronounced seasonal changes in the morphology of androgen-sensitive song tissues that may be due directly to androgens or indirectly via androgen effects on afferent song structures (Brenowitz and Lent, 2001), essentially readying or activating the vocal circuit. Without circulating androgens, the vocal system cannot be activated even if vocal circuit connections, vocal motor neuron number, and larynx size and physiology are all in place.

PBDE-209 treated male *X. laevis* had significantly smaller diameter vocal motor neurons than control male *X. laevis.* These differences may represent disruption of

activational androgen levels. PBDE treatment of mice has resulted in lasting anti-androgenic effects. Neonatal male mice treated with the PBDE congener, PBDE-99, also commonly used in consumer products, showed feminization of male-typical behaviors that continued into adulthood. Accompanying the behavioral changes were also reductions in androgen levels in treated mice (Lilienthal et al., 2006). Reductions in androgen levels, but also changes in the vocal tissue androgen binding capabilities or androgen sensitivity may also account for reduced N. IX-X diameter. Stoker et al. (2005) noted that some PBDE congeners block androgen-induced transcriptional activation of the androgen receptor while other PBDE congeners competitively bind with androgen for the androgen receptor. Both means of disruption would certainly limit the typical activation events in the *X. laevis* vocal circuitry.

Reduction in neuron size in PBDE-treated *X. laevis*, was accompanied also by reduction in n. IX-X neuron number. These data, though significant, are a little more difficult to reconcile than reductions in n. IX-X neuron size. The quantity of n. IX-X neurons is thought of as an organizational effect. By metamorphosis (PM0), males and females have the same number of n. IX-X neurons (Hannigan and Kelley 1986). With the masculinization of peripheral vocal structures, there is also an addition of connections from n. IX-X to *m. dilator laryngis*, but no net addition of n. IX-X neurons. By the time *X. laevis* reaches PM6, there are marked sex differences in n. IX-X neuron number not because of the net addition of neurons, but because of programmed neuron death (Simpson et al. 1986). Circulating androgen, protect from the net loss of n. IX-X neurons in developing males (Wetzel et al. 1985). At sexual maturity, the male n. IX-X contains

an average of 1220 motor neurons, while the female has only 760 motor neurons (Hannigan and Kelley 1986).

The final number of n. IX-X neurons is thought to be permanent after they are organized during the developmental critical period. After the critical period, when n. IX-X number is supposedly set in stone, not even castration will change the cell count (Wetzel et al. 1985). In the present study, though, n. IX-X populations were reduced after PBDE-209 treatment. One possible explanation for the reduction in n. IX-X neuron number may simply be PBDE-209's neurotoxicity. He et al. (2007) showed that hippocampal neurons treated with PBDE-47 showed signs of oxidative stress and apoptosis, but only at the high dose, 41.2 μM. These data show that exposure to PBDE may have neurotoxic effects at certain threshold levels, but in the case of PBDE-47, not at concentrations below 41.2 μM.

Evident in other studies are modifications in behavior that could be the effects of neurotoxicity. For instance, mice treated perinatally with relatively high concentrations (0.6, 6, or 60 mg/kg/day) PBDE-99 showed behavioral alterations, including hypoactivity and thigmotaxis, a sign of anxiety in mice (Branchi et al., 2002). Environmentally relevant levels of PBDE-99 (60 and 300 ppb) are also able to alter behavior, feminizing male-typical behaviors in offspring of treated rat mothers (Lillienthal et al. 2006). Likewise, commercial PBDE mixtures can hinder intracellular signaling processes in the brain that are associated with neuron development and learning and memory (Kodavanti and Ward, 2005).

PBDE mixtures have demonstrated effects on basic developmental programs and also target endocrine-related processes. The present study strengthens the evidence that

flame-retardant congeners, at concentrations similar to environmental levels, have the ability to alter brain tissue in adults. Coincidentally, these tissues that are essential for reproduction in *Xenopus laevis*, are also androgen sensitive. In order to successfully attract mates, male *X. laevis* must maintain precise levels of androgens to activate both the peripheral tissue andcentral nervous circuitry associated with mate vocalizations. Vocal system activation is governed by androgens and signals the onset of seasonal tissue growth. An absence of androgen levels or androgen binding ability may, therefore, slow or stop seasonal activation altogether and the localized cell growth that accompanies activation. Studies on the functionality of the vocal system in PBDE-209 treated adult frogs will help to explain the ultimate effects of these neuronal deficits. Neuronal deficits may ultimately affect the ability for a male to successfully attract mates and reproduce.

CHAPTER 6
VOCAL DEFICITS IN MALE *Xenopus laevis* AFTER PBDE-209 TREATMENT

Reproductive success depends on proper communication between prospective mates. Vocal signals are an especially critical aspect of mating behavior in the African Clawed Frog, *Xenopus laevis*. Male *X. laevis* advertise their interest with a rapid series of oscillating click vocalizations. Interested females answer with rapid ticking sounds (rapping), and uninterested females will shun males using a slow series of tick vocalizations (Tobias and Kelley, 1995; Kelley, 1986). Without these mating vocalizations, reproduction could not take place.

Vocal system anatomy begets these differences in vocal behavior. The adult *X. laevis* vocal system is sexually different. Male typical vocalizations rely on the precise organization of vocal circuitry as well as the appropriate levels and actions of androgens. Vocal structures depend on precise levels of androgens for proper tissue organization and function (Kay et al., 1999). Appropriate levels of androgens establish these sex differences by the time the frog reaches sexual maturity. The developing vocal system is especially sensitive to androgens for a critical period that begins at metamorphic climax and subsides six months after metamorphosis. At this point, *X. laevis* is sexually mature, and males display both morphological and physiological sex differences in the vocal system (Kelley 1986).

At sexual maturity, both male and female *X. laevis* are capable of vocalizing, though their calls show measurable sex differences. Signals from n. IX-X initiate the contraction of the laryngeal dilator muscles. The contracted muscles pull apart the bell

clapper-shaped arytenoid laryngeal cartilages. Relaxation of the muscles allows the cartilages to snap back together, making a clicking or ticking vocalization (Tobias and Kelley, 1995). Males have fast twitch laryngeal muscle fibers and weak laryngeal motor nerve (N. IX-X) to laryngeal dilator muscle synapses that require constant neuromodulation. The resultant vocalizations consist of rapid clicks of oscillating amplitude. Females have slow twitch laryngeal dilator muscles and estrogen-mediated strong synapses, giving way to slower ticks of relatively the same, small amplitude (Moore et al., 2005).

Common female and male call types recorded by EMG are illustrated in Figure 25, and include: 1A) Female "rapping," a call typical to reproductively ready females consisting of long, low amplitude fast click trains, 1B) male "growling," lengthy, rapid trains of relatively low amplitude clicks, and 1C) male "advertisement", a rapid series of clicks that are produced in short bursts and are of vastly varied amplitude. Distinguishing female typical from male typical calls is quite obvious, noting the differences in click amplitude and the cadence with which the clicks are produced. Also easily recognizable, is how vocal system anatomy and physiology directly represents the produced vocal behavior, especially in the precisely organized male vocal system.

Specific developmental programs and appropriate androgen levels during the developmental critical period are essential for the proper formation of the *X. laevis* vocal system. For the adult male, these developmental programs will ensure the proper vocal equipment for attracting mates. Androgen levels are equally as critical for male-typical call production and the maintenance and function of the vocal circuitry as they are for building the vocal system. There exists, however, some hormone-related plasticity in the

Figure 25. Electromyograms (EMGs) of typical call types from *Xenopus laevis* adults. All EMGs were recorded during the experimental period. A. Female "rapping" vocalization indicates reproductive readiness. Notice the slow click vocalizations of similar short amplitude. B. Male growling consists of clicks of similar high amplitude. C1-2. Male advertisement calls. Note the rapid clicks and variation in amplitude.

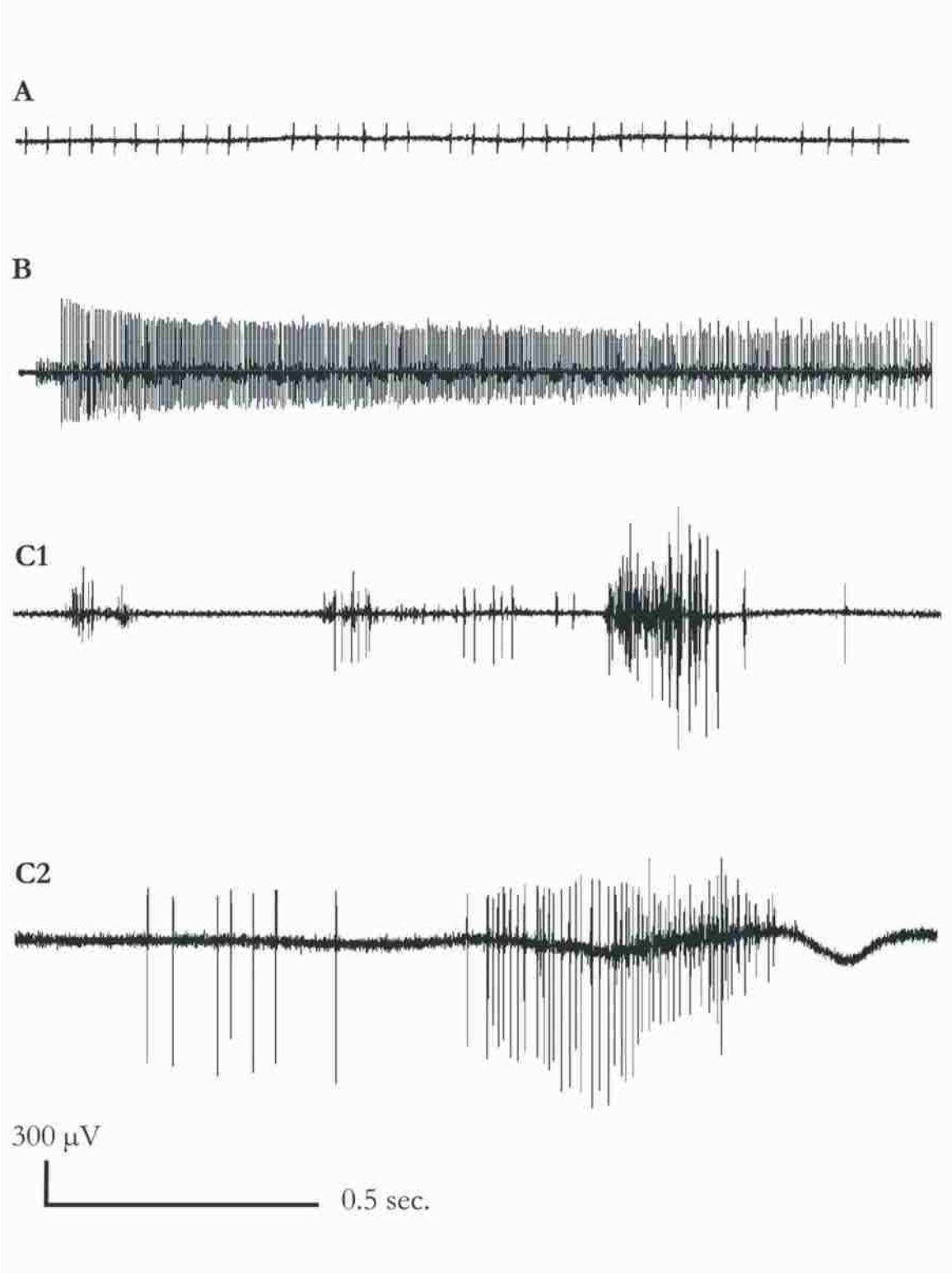


Figure 25

vocal system. Juvenile females with testis transplants, for example, were later able to make male-typical calls in adulthood (Watson and Kelley, 1992). After androgen-exposure, 22% of the treated adult females elicited masculinized click trains that were faster than typical female calls but still slower than male calls (Hannigan and Kelley, 1986). Castrated adult males were also able to regain calling ability after testis transplantation (Watson and Kelley, 1992). Call production in males is thus androgen dependent, and though females are capable of vocalizing, only treatment with exogenous androgens during the developmental critical period will allow the frog to produce the rapid male-typical calls.

EMGs (Figures 26-29) illustrate portions of vocal episodes recorded from representative specimens. In order to appreciate differences in click amplitudes or variety in call type, EMGs were first compressed to show a multitude of calls per time scale then, slowed 10X to observe a single call, and finally slowed another 10X to show specific details of the call. Both control and low dose group frogs elicited typical advertisement calls with clicks of varying amplitude. Common among control frogs were extremely long growling episodes interspersed with shorter advertisement trills (Figure 5A-B). Control males did not call as frequently as low or middle dose frogs, but their calling episodes lasted much longer due to the extent of their growl/advertisement vocalizations.

Like control frogs, typical advertisement calls with high amplitude clicks were recorded from low dose frogs. Most low dose frogs produced EMGs characteristic of multiple burst advertisement calls. Representative EMGs (Figure 27) show that most low dose frogs' advertisement calls consisted of a pair or a triplet of short click bursts. Each

Figure 26. Typical EMG recordings from control male frogs. EMGs were recorded during overnight recording sessions lasting a minimum of three hours. Bipolar electrodes were sutured into laryngeal dilator muscle, and electromyographic signals were recorded via the recording apparatus described in Figure 4 (Low pass filter = 300 Hz, High pass filter = 10 Hz, Gain = 10,000 X, sampling rate of 0.05 seconds/division and amplitude range of +/- 3000 μV). Control animals reliably elicited advertisement calls consisting of rapid clicks of oscillating amplitude. A1. One example of an advertisement call with short bursts of high amplitude clicks. A2. Another control frog advertisement call marked by long growling episodes consisting of rapid, similar amplitude clicks interspersed with short bursts of high and variable amplitude clicks. B-C. Recordings are slowed 10x each to reveal subtle differences in click amplitude and frequency.

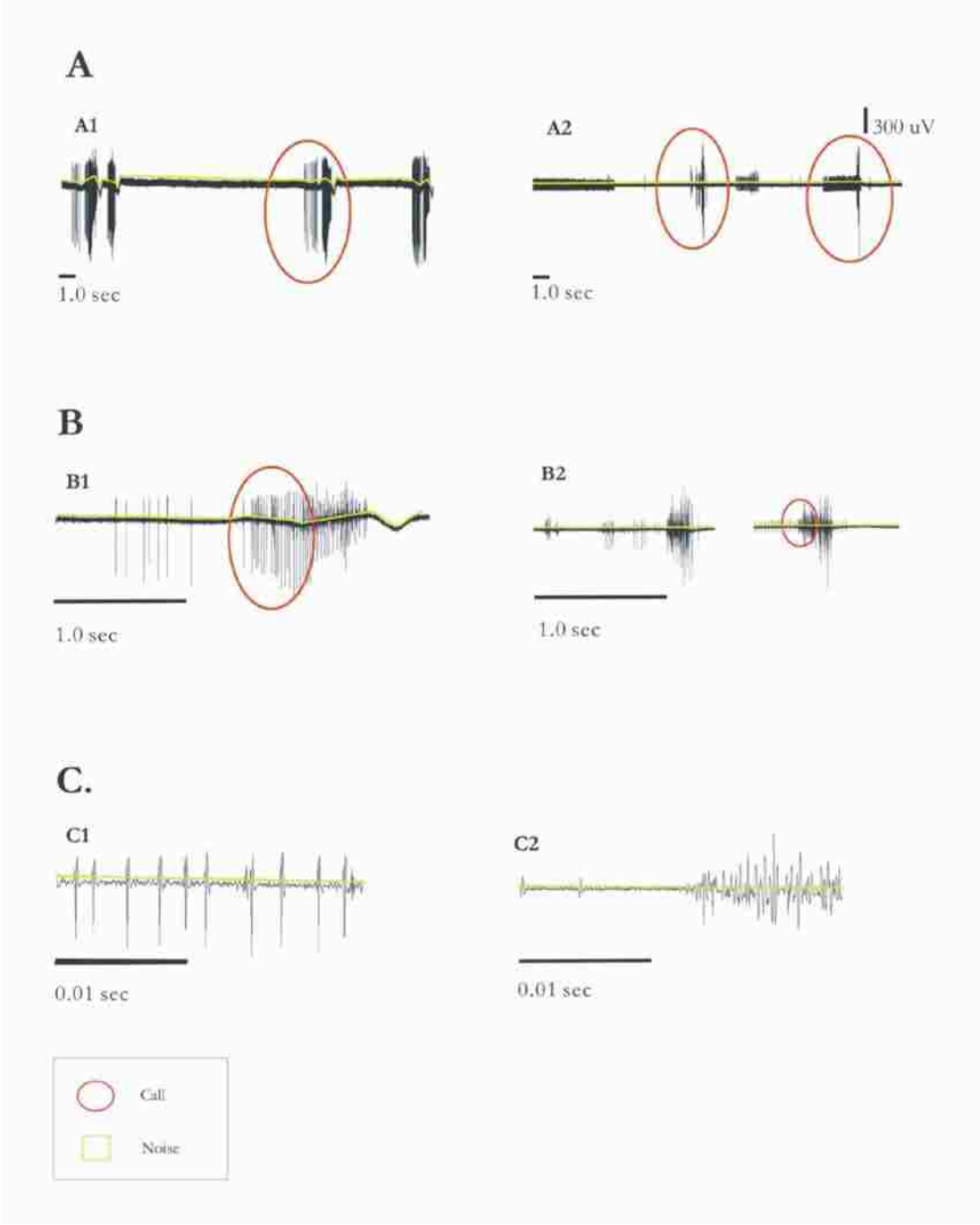

A
A1
1.0 sec
A2
300 uV
1.0 sec
B
B1
1.0 sec
B2
1.0 sec
C.
C1
0.01 sec
C2
0.01 sec
Call
Noise

Figure 26

Figure 27. EMG recordings from low dose male *X. laevis* (Low pass filter = 300 Hz, High pass filter = 10 Hz, Gain = 10,000 X, sampling rate of 0.05 seconds/division and amplitude range of +/- 3000 μV). Like the control frogs, low dose frogs reliably elicited advertisement calls but did not growl. Typical calls consisted of short burst trains of variable high amplitude clicks.

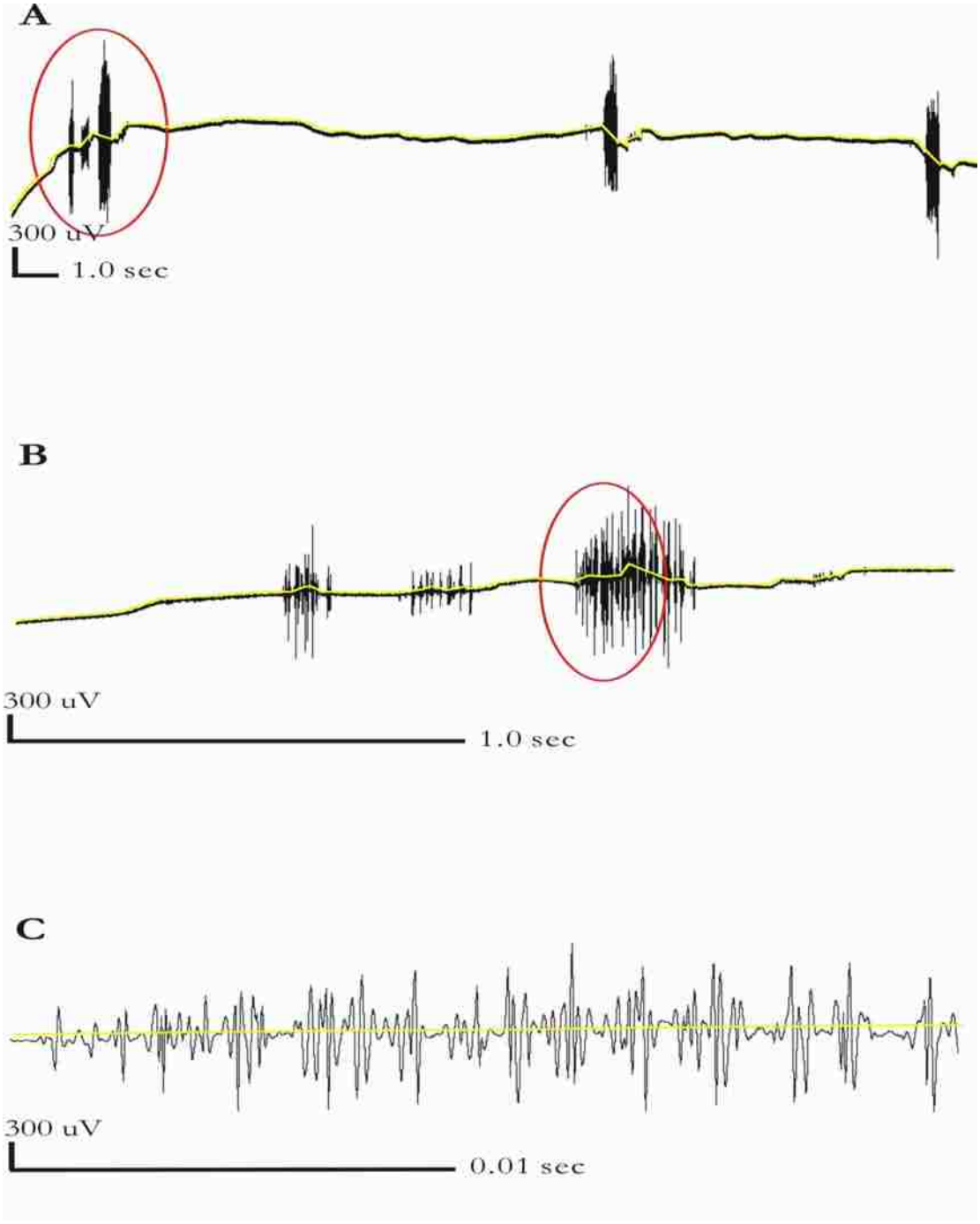


Figure 27

burst contained several high amplitude clicks. Though these short trills were also seen in control animals, the bursts were most often accompanied by growling episodes that were absent in low dose males. Vocalizations were significantly smaller in amplitude than control ($p < 0.001$) and low dose frogs ($p < 0.001$).

The differences in call performance increased further with increases in PBDE-209 concentration. Representative EMGs from the middle dose group showed that some frogs produced typical-looking male advertisement calls (Figure 28A), while other frogs from this group produced short duration, and weak-amplitude clicks (Figure 28B). When average click amplitude for the middle dose groups was compared to click amplitude in control and low dose EMGs, it was actually much lower, suggesting a reduction in the ability to produce high amplitude clicks.

Call parameters were reduced even more in the high dose and positive control frogs compared to each of the other treatment groups. Inspection of representative EMGs from high dose frogs (Figure 29) shows a shift from typical advertisement vocalizations to the production of very short bursts of similar-sized weak amplitude clicks. High dose frogs had the smallest amplitude and least frequent calls among all the treatment groups.

A closer look at the ranges in click amplitude in each treatment group (Figures 30-31) reveals that there was significant variation in amplitude range in all groups, but the control and low dose frogs had wider ranges and higher amplitudes overall. Control frogs clicked amplitudes ranging from 60-6660 μV, and the amplitudes of low dose frog clicks fell between 240-5520 μV. These two groups varied little from one another, but there were vast differences between the control and low dose groups and the middle and high PBDE-209 dose frogs. Though middle dose frogs were able to produce variable

Figure 28. EMG recordings from middle dose male *X. laevis* . (Low pass filter = 300 Hz, High pass filter = 10 Hz, Gain = 10,000 X, sampling rate of 0.05 seconds/division and amplitude range of +/- 3000 μV). A1. Example of what appears to be a typical male advertisement call. Click amplitude measurements, however, were much lower in middle dose frogs compared to control and high dose frogs. A2. Another example of a middle dose frog EMG for which frequent short burst calls of low amplitude were recorded. A-C. Recordings are slowed 10x to show subtleties in click amplitude and frequency. Though advertisement calls appear to be similar to control or low dose frogs, middle dose frogs had calls of smaller amplitude than low dose and control frogs.

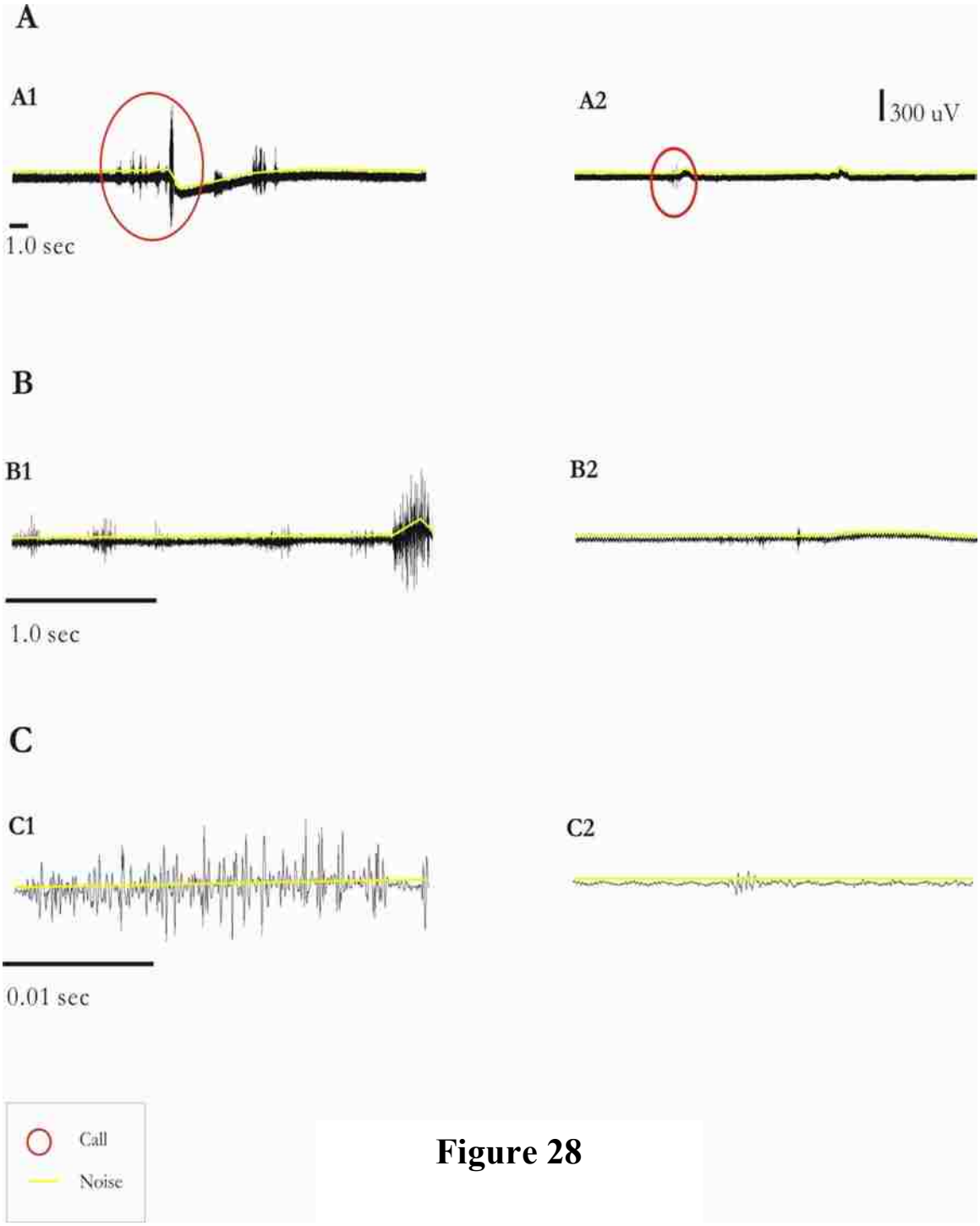


Figure 28

Figure 29. EMG recordings from high dose PBDE-209 treated *X. laevis* (Low pass filter = 300 Hz, High pass filter = 10 Hz, Gain = 10,000 X, sampling rate of 0.05 seconds/division and amplitude range of +/- 3000 μV). High dose frogs had uncharacteristically fewer and smaller calls compared to frogs in the other treatment groups. A. Calls appear to be short bursts of extremely low amplitude clicks. As recordings are slowed, B-C, typical high dose calls are seen to consist of short 4-5 click bursts. Clicks in the high dose group averaged only 60 μV in height.

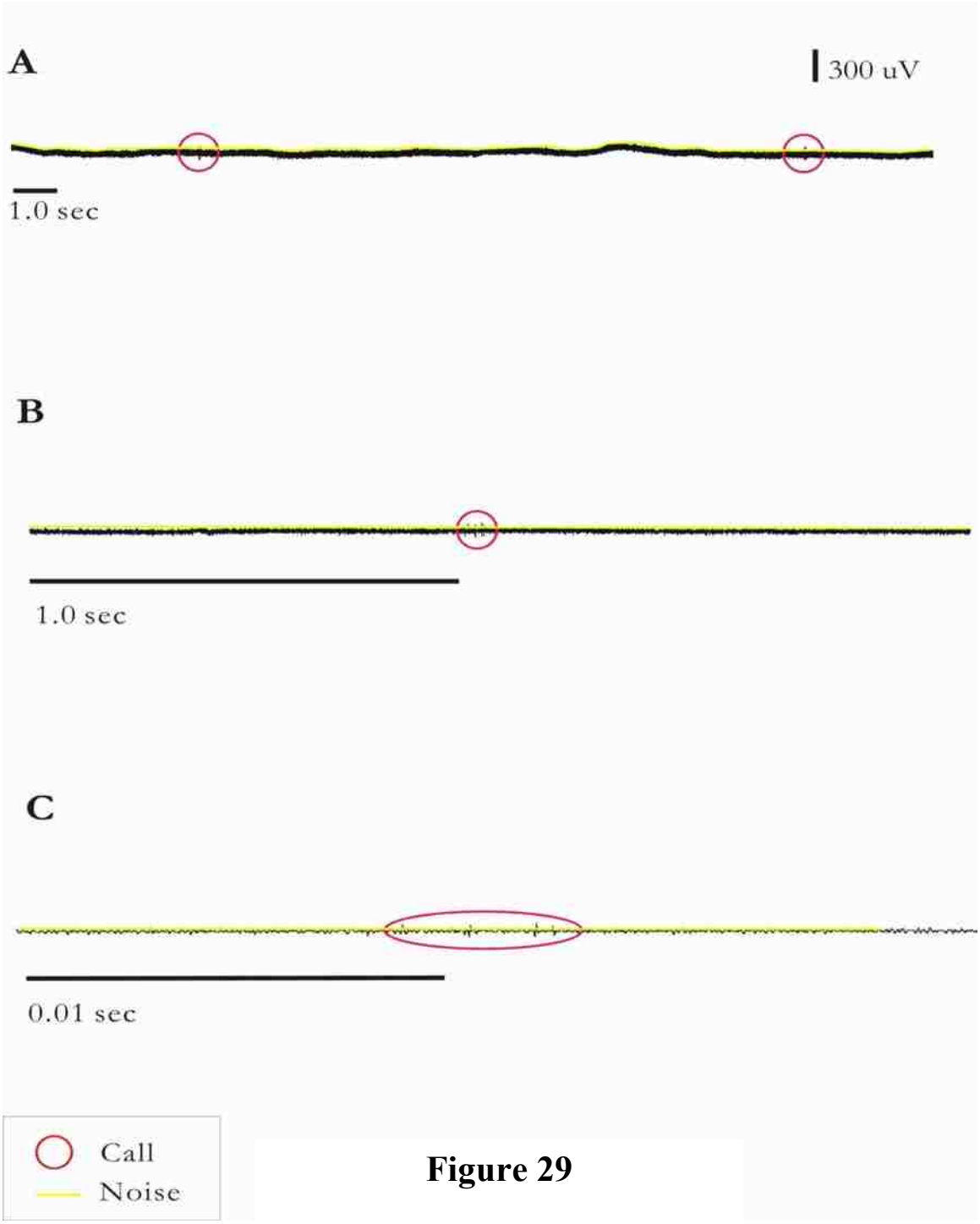


Figure 29

Figure 30. Representative EMG traces from: A. control and B. low dose frogs. Scatter plots in the second row indicate the frequency of calls for each frog recorded for control, low dose frogs during the experiment. Histograms in the third row indicate ranges in click amplitude. Control and low dose frogs called the most overall and retained the ability to make calls with clicks of widely ranging amplitude after PBDE-209 treatment.

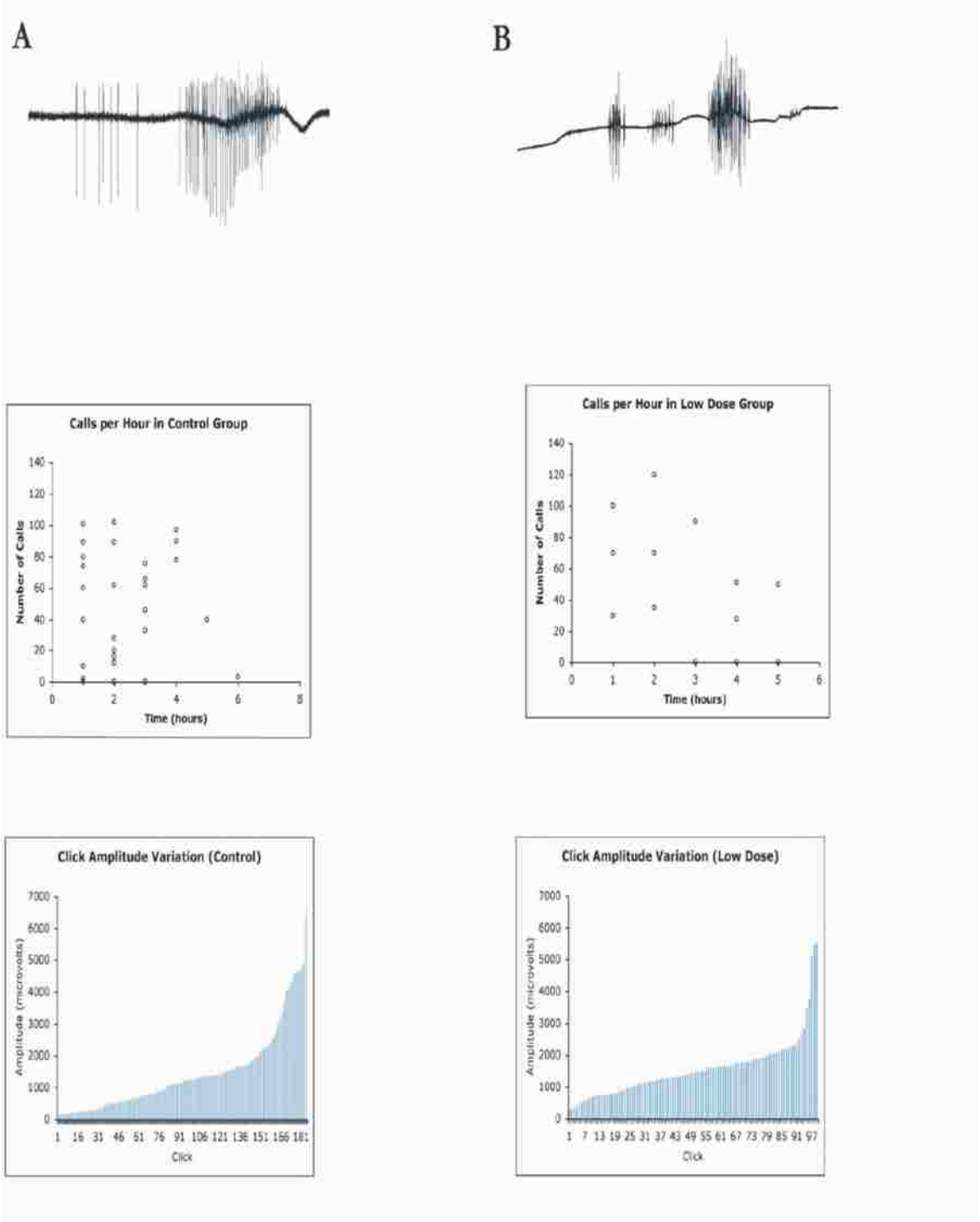
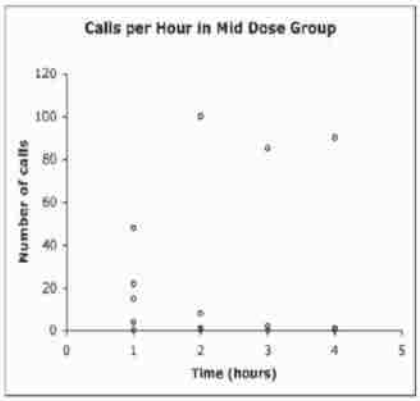
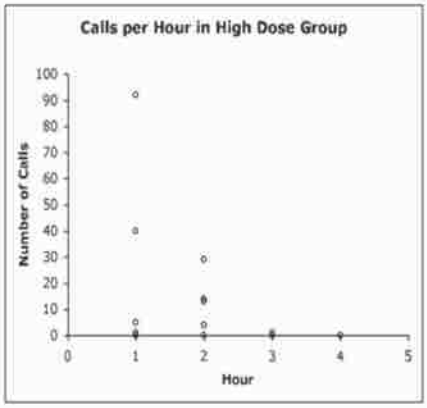
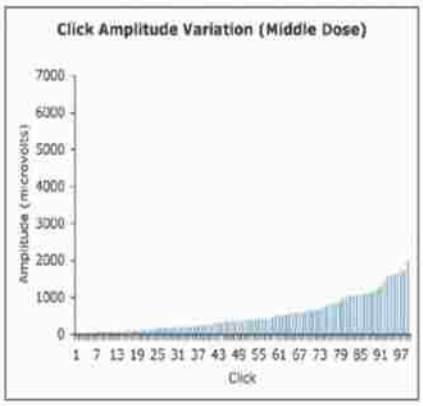

A
B
Calls per Hour in Control Group
Number of Calls
Time (hours)
Calls per Hour in Low Dose Group
Number of Calls
Time (hours)
Click Amplitude Variation (Control)
Amplitude (microvolts)
Click
Click Amplitude Variation (Low Dose)
Amplitude (microvolts)
Click

Figure 30

Figure 31. Representative EMG tracings from: A. middle dose and B. high dose frogs. Scatter plots in the third row show the number of calls per hour for high dose and middle dose frogs. Histograms indicate ranges of click amplitudes that fell significantly below control, low dose, and middle dose click amplitudes. Middle dose frogs had lower than average click amplitudes, as did high dose frogs that also failed to produce clicks of widely ranging amplitudes after treatment.


Calls per Hour in Mid Dose Group
Number of calls
Time (hours)


Calls per Hour in High Dose Group
Number of Calls
Hour


Click Amplitude Variation (Middle Dose)
Amplitude (microvolts)
Click

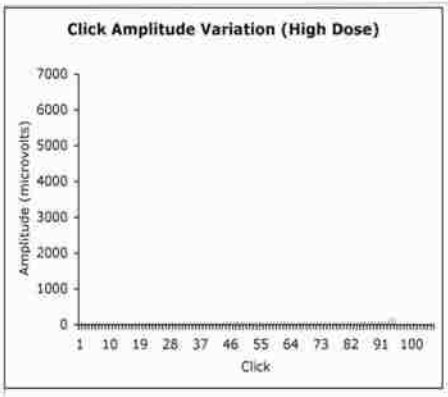

Click Amplitude Variation (High Dose)
Amplitude (microvolts)
Click

Figure 31

amplitude clicks, the range in click amplitude (30-960 μV) was significantly lower than control and low dose groups but higher than high dose (30-210 μV) frogs. Recall that one of the characteristics of the male advertisement call is its wide range in click amplitude. Coefficients of variation (CV) were calculated for the click amplitude ranges of each treatment group. High CV indicates high variation in the sample. In the control group, CV for click amplitude was 0.896. CV was also high for low dose frogs (0.669). For middle dose frogs, the CV was higher than in low dose frogs (0.779), probably because of the variation between typical advertisement-like calls and small amplitude short burst calls. Though CV was high in middle dose frogs, the average click amplitude was still smaller than control and low dose frogs. High dose frogs had the lowest CV (0.515), which was expected as most every EMG recorded from high dose frogs had short burst calls with clicks that did not vary much in their short amplitude. Both the anatomy of n. IX-X and the rest of the vocal circuitry, as well as the physiological differences in the vocal system allow for males to produce rapid click trains with clicks that can range from 30-7000 μV tall. Alterations in the ability for a male frog to produce calls ranging widely in click amplitude indicate disruption in vocal physiology (Figure 32).

Average call duration varied greatly among treatments, most likely due to the short vocalization bursts that were quite frequent and rarely three or more seconds apart interspersed with long advertisement click trains characteristic of control and low dose frogs. Middle dose frogs produced the longest calls but not significantly longer than control and low dose frog calls. High dose and positive control frogs had significantly shorter duration calls than those of control, low dose, and middle dose frogs. For the most part, frogs called for the entire minimum treatment period, but the total number of

Average Click Amplitude in PBDE-209 Treated Adult Male Frogs

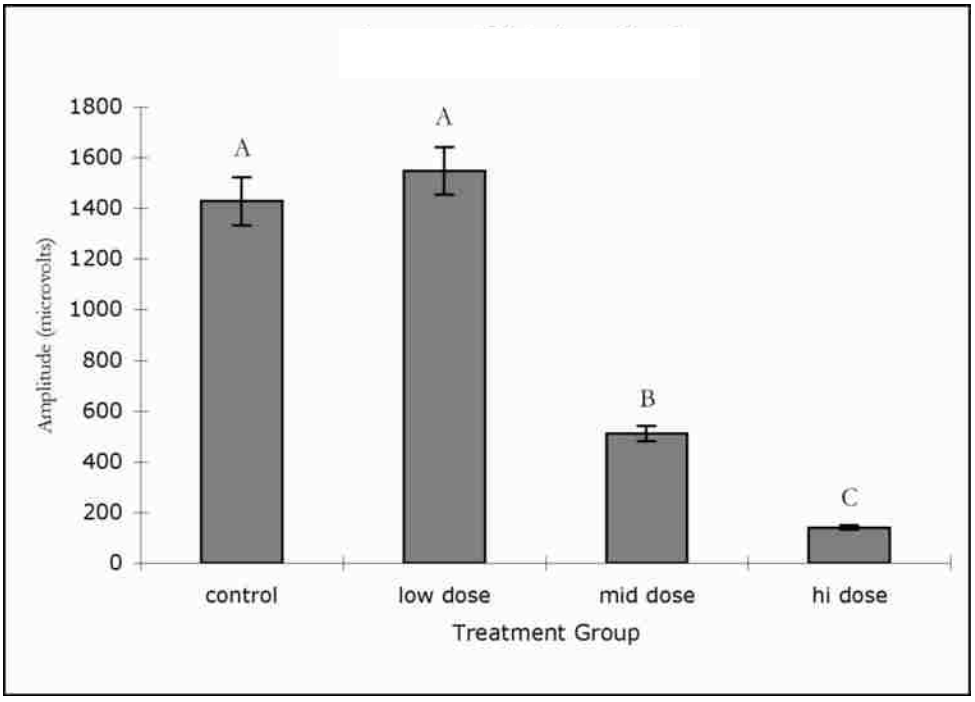


Figure 32

Figure 32. Comparison of average click amplitude among treatments. Though there was no difference in average click amplitude between control and low dose male *X. laevis*. Average click amplitude was significantly different between these two groups and the middle dose frogs. As PBDE-209 concentration increased, the average click amplitude decreased. There were also significantly lower amplitude clicks in the high dose frogs.

Total Number of Calls by PBDE-209
Treated Male *X. laevis*

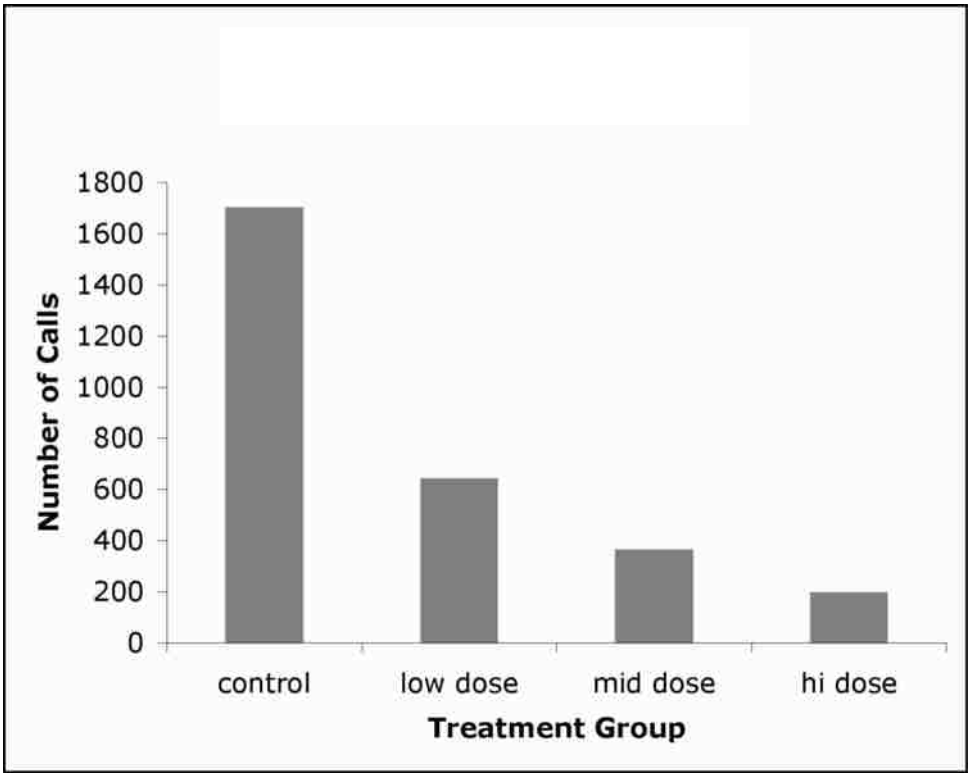


Figure 33

Figure 33. Total number of calls per treatment. Just as the ability to make male-typical calls diminished with increasing PBDE-209 concentration, so did the number of calls per treatment. Control frogs called the most (1703). The number of calls decreased as PBDE-209 concentration increased: Low dose frogs made 644 calls, middle dose frogs made 366 calls, and high dose frogs made 199 calls.

calls decreased as PBDE-209 concentration increased (Figure 33**)**. For a male *X. laevis* to make an effective call, it relies on an attractive advertisement consisting of rapid click vocalizations of varying amplitude. With PBDE-209 exposure, however, the ability for middle and high dose treated male frogs to call was severely disrupted.

Discussion

Often hormonally constructed anatomy is fixed once it is built during the developmental critical period. Generally, this dogma is true for most vertebrate reproductive tissues that are later put into action by the very same hormones that built the organs. During the developmental period of reproductive organ building, specific levels of sex hormones as well as proper tissue building are essential such that appropriate function and behavior will result in adulthood.

The vocal system of male *Xenopus laevis* is one organ group that relies on precisely formed anatomy and specific levels of androgens for proper function. During the developmental critical period, androgens establish measurable sex differences in peripheral vocal structures as well structures in the brain that mediate reproductive vocal behavior. The structure of the tissue is directly indicative of the resultant behavior. All the tissues in the vocal system contain androgen receptors, and they are all subject to the seasonal influences of circulating endogenous hormones (Schmidt, 1984; Wetzel et al. 1985). Androgens build the circuitry, ready it for mate vocalization, and activate its function. Reproductive readiness in the male includes the growth of the larynx and laryngeal nerve (Sassoon et al., 1987), as well as the growth the vocal motor nucleus (n.

IX-X) itself (Hannigan and Kelley 1986). Androgen-mediated reproductive growth allows the frog to elicit rapid, variable high amplitude male-typical calls.

It is clear that proper vocal anatomy is crucial for the production of attractive vocalizations and that androgens are the critical directors of vocal system form and function. When Hannigan and Kelley (1986) exposed adult female frogs to androgens, they were able to masculinize a portion (22%) of the females' calls. The masculine vocal behavior was incomplete, however, as the calls were never as rapid and as high amplitude as typical male calls, indicating as well that the proper anatomy is necessary for male-typical vocalization. Castrated males, though, were able to retain their calling ability with androgen treatment (Wetzel et al., 1985). Only androgen exposure during the developmental critical period can fully masculinize the vocal system and the resulting calls in adulthood.

In the present study, the putative anti-androgenic PBDE-209 reduced male-typical calling ability in treated frogs, nearly eliminating calls in the high dose group. As PBDE-209 concentration increased, the number of calls as well as call amplitude decreased. Other congeners in PBDE-209's family are known to feminize behavior in rodents that were treated *in utero* (Lilienthal et al. 2006). Feminization by PBDE-209 provides a plausible explanation for the absence of typical ticks in larger-dose treated frogs. The anti-androgenic positive control also provided representative evidence that PBDE-209 may be anti-androgenic, as the EMG records show that high dose frogs produced calls of similar number, amplitude, frequency, and duration.

Deficits in calling ability and destruction of the vocal system anatomy are not direct indicators of the mechanism of PBDE-209's disruptive action. The experimental differences only demonstrate that change has taken place in PBDE-209 treated frogs. If PBDE-209 is acting as an anti-androgen, there are many possible ways it can influence the vocal system, especially because the entire system contains androgen receptors and relies on androgens to function properly. Reductions in androgen levels, but also changes in the vocal tissue androgen binding capabilities or androgen sensitivity may also account for behavior change. Stoker et al. (2005) noted that some PBDE congeners block androgen-induced transcriptional activation of the androgen receptor while other PBDE congeners competitively bind with androgen for the androgen receptor. Either androgen-blocking events would negatively impact male calling.

The current study supports the notion that PBDE-209 acts as either an anti-androgen or neurotoxin that negatively impacts vocal function. Anatomical studies in the previous chapter indicate that PBDE-209 not only limits vocal function, but it also negatively impacts n. IX-X neuron size and number. PBDE-209 treatment severely reduced the ability for frogs to call, which will ultimately reduce reproductive success. Without the vocalization, males cannot attract females to mate. How, then, might alterations in vocalization impact species survival? Of further concern, however, is how this relatively ubiquitous group of pollutants might influence other hormone-mediated functions, especially in humans. Not only have PBDEs been measured in our air, soil, and water, but they have made their way into our bodies. PBDE levels in breast milk and body fat are a concern, too, for humans growing through their developmental critical periods. Because developmental programs are disrupted by PBDEs, the effects of PBDE-

mediated disruption can continue to affect an animal into adulthood, affecting both morphology and behavior. The impact of these putative anti-androgens may well be handed down to the next generation.

CHAPTER 7
CONCLUDING REMARKS

Human impact on the earth has severely compromised species survival. Specifically, habitat destruction and man-made pollutants critically threaten reproductive success and challenge an animal's ability to respond to stress events. The resultant decline in population numbers is most evident in the amphibia for which severe population reductions and even extinctions have become apparent since the 1980s (Colborn et al. 1994). The two major extinction events in the late 1980s were not punctuated events, rather, evidence suggested steady, worldwide declines in amphibian numbers (Guillette et al. 1997). These events initiated the struggle to pinpoint the cause of amphibian declines. Though a solitary cause cannot be identified as the main agent of decline, the consensus maintains that synergy among individual stressors can devastate populations.

Habitat destruction remains the obvious detriment, but amphibians face more discreet but steady threats from anthropogenic pollutants. Long before scientists applied themselves to studying pollutant effects, Rachel Carson's Silent Spring (1964) warned of the dangers of pesticide overuse. Later, as not only disappearing numbers of species became apparent, reports emerged of populations encountering stunted growth, severe morphological deformities, overwhelming infections, and reproductive and behavioral abnormalities. Frog larvae (*Xenopus laevis* and *Rana pipiens*), when exposed to a mixture of pesticides each at a low concentration took longer than controls to

metamorphose, and when compared to controls, the slow metamorphosing frogs were smaller (Hayes et al. 2006). As wildlife is exposed, not only to pesticides but to a cocktail of agricultural, industrial, and waste chemicals, they face challenges not only in development, but also in reproduction and ultimately survival.

Though Hayes et al. (2006) demonstrate a measurably greater detriment to larval development when chemicals work in synergy, there still remains much work to be done on the toxicity of individual chemicals. Several agricultural, industrial, and waste chemicals are known to directly disrupt the endocrine system. Because hormones tightly govern growth, metabolism, response to stress, and reproduction, any disruption of normal hormone levels can critically impact reproduction and survival.

Hormone levels are especially important during two critical periods: a developmental organizational period and an adult activational period when maintenance and seasonal trophic effects necessitate hormone influence on reproductive organs. Exposure to environmental toxicants during the vertebrate organizational period severely impacts organ formation. The developmental critical period is marked by the sexual differentiation of the gonads, areas of the brain that govern mating behaviors, and peripheral secondary sex structures like the vocalization structures or nuptial pads in frogs. Once these sexually different structures are formed, they are generally rigidly set with physiological and coincidental morphological changes following seasonal cycles during which steroid hormones activate reproductive tissues for breeding. Because many disrupting agents are so hormonally active, exposure during reproductive activation can hinder an animal's ability to elicit proper mating behaviors, engage in mating, and

produce fit gametes and offspring. Any challenges to a species' reproductive effort will detriment reproductive fitness and ultimately, species survival.

The vocal system of male *Xenopus laevis* served as an ideal model for testing the effects of a putative anti-androgen, PBDE-209. The male vocal system is built by androgens during development, and during adulthood, circulating androgens maintain the system and mediate seasonal growth. In the present study, critical period-specific effects of PBDE-209 were monitored by exposing both developing and adult *X. laeivis* to environmentally relevant levels of PBDE-209. PBDE concentrations used in this study are relatively conservative compared to levels found in some environments, and fall within the lower ranges of PBDEs found in wildlife and human tissues. Thus, the anti-androgenic effects observed in both juveniles and adults provide alarming evidence of the possible endocrine disrupting and possibly cytotoxic effects of PBDEs, considering especially the comparatively higher concentrations our bodies stockpile.

PBDEs are everywhere. Not only are they found in expected places like garbage and salvage areas and in sewage runoff, but PBDEs are also found in our clothing, household electronics, food, and house dust. It is detected in river sediment and in marine mammals, and in alarmingly increasing concentrations in humans, especially children. Evidence is also increasing developing organisms may be sensitive to PBDE's endocrine disrupting effects.

In this study, juveniles treated with PBDE-209 had fewer n. IX-X neurons, and the neurons that remained were smaller than those of untreated frogs. Adult *X. laevis* rely on a vocal system with many large n. IX-X neurons in order to procure vocalizations that attract females. Females can vocalize, but their comparatively smaller and fewer n. IX-X

neurons limit vocalizations to slow and small clicks. Had these experimental juveniles been allowed to mature to a breeding season, their vocalization efforts would be impaired.

Likewise, treatment with PBDE-209 had anti-androgenic effects on adult frogs. Contrary to the hypothesis, PBDE-209 treatment affected n. IX-X cell number and size. With increasing PBDE concentration, there was a decrease in cell number and size. Because previous research (Kelley, 1986; Wetzel and Kelley, 1983; Perez and Kelley, 1996; Kay et al. 1999) has shown that n. IX-X is relatively resistant to changes in neuron population numbers in adulthood, no changes were expected in the present study. Not only were there changes in neuron number, but size also decreased with increasing PBDE-209 concentration.

Vocal function was also compromised in adults. Call frequency decreased with increasing PBDE concentration as did click amplitude. The androgen-mediated anatomy of the male vocal system is built to produce rapid click calls of varying amplitude. Trademarks of typical male calls are clicks of oscillating amplitude made in rapid succession. Middle dose and high dose PBDE-209 treated males could not make high amplitude clicks. High dose frogs had short, small amplitude vocalizations much like the anti-androgen control-treated frogs.

PBDE-209 effects were not critical period-restricted, rather anti-androgenic effects were evident in both developing and adult frogs. Effects were also not limited to changes in anatomy but PBDE-209 also affected physiology and behavior in adult frogs. PBDE-209 treated frogs could not communicate properly, adding evidence to the growing concern over PBDE's effects on all organisms.

The present study provides an excellent starting point for future research. As I continue my research, I would like to employ new methods to test more precisely the mechanism of action of PBDE-209 on the vocal system. There are many routes through which an endocrine disruptor may affect a hormone-dependent system both during developmental critical periods and during adulthood. Future research projects will include assays of androgen and androgen receptor levels in n. IX-X, and later studies of how PBDE-209 might be disrupting the communication of androgens with the vocal system. Generational studies will also be essential in determining the impact of PBDE as a hand-me-down chemical.

The impact of PBDEs on human health is increasing as chemical levels increase in humans and their surroundings. Research regarding endocrine disruptor effects on developing young is especially critical as we find that children may be shouldering the highest chemical body burdens. More worrisome is that a portion of the burden may be handed down from mother to child during gestation and nursing, possibly impacting young during the most critical periods of development.

LITERATURE CITED

Branchi, I., E. Alleva, L. G. Costa. 2002. Effects of perinatal exposure to a polybrominated diphenyl ether (PBDE 99) on mouse neurobehavioural development. Neuro. Tox. 23(3): 375-384.

Brenowitz, E.A. and K. Lent. 2001. Afferent input is necessary for seasonal growth and maintenance of adult avian song control circuits. J. Neurosci. 21: 2320-2329.

Carson, R. 1962. Silent Spring. Boston, Houghton Mifflin. 378.

Chen D., B. Mai, J. Song, Q. Sun, Y. Luo, X. Luo, E.Y. Zeng, R.C. Hale. 2007. Polybrominated diphenyl ethers in birds of prey from Northern China. Environ. Sci. Technol. 41(6): 1804-1805.

Colborn, T., D. Dumanoski, J.P. Myers. 1996. Our Stolen Future : How We Are Threatening Our Fertility, Intelligence and Survival. New York, Dutton. 306.

Costa L.G., G. Giordano. 2007. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. Neuro. Tox. 28 (6): 1047-1067.

Darnerud, P.O., G.S. Eriksen, T. Jóhannesson, P.B. Larsen, and M. Viluksela. 2001. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. Environ. Health Pers. 109(Suppl 1): 49–68.

de Boer, J., P.G. Wester, A. van der Horst, and P.E.G. Leonard. 2003. Polybrominated diphenyl ethers in influents, suspended particulate matter, sediments, sewage treatment plant and effluents and biota from the Netherlands. Environ. Poll. 122(1): 63-74.

Duellman, W. and L. Trueb. 1984. Biology of Amphibians. Kansas, JHU Press. 670.

Gorlick, D. and D.B. Kelley. 1987. Neurogenesis in vocalization pathway of *Xenopus laevis.* J. Comp. Neurol. 257: 614-627.

Guillette, L.J., Jr., D.A. Crain, A.A. Rooney, and A.R. Woodward. 1997. Effect of acute stress on plasma testosterone, estradiol-17ß and corticosterone concentrations in juvenile alligators living in control and contaminated lakes. J. Herpetol, 31: 347-353.

Guvenius, D.M., A. Aronsson, G. Ekman-Ordeberg, A. Bergman, and K. Noren. 2003. Human prenatal and postnatal exposure to polybrominated diphenyl ethers, polychlorinated biphenyls, polychlorobiphenylols, and pentachlorophenol. Environ. Health Pers., 111(9): 1235-1241.

Hannigan P., D.B. Kelley. 1986. Androgen-induced alterations in vocalizations of female Xenopus laevis: modifiability and constraints. J. Comp. Physiol. [A]. 1986 158(4): 517-527.

Hayes, T.B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A.A. Stuart, A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. PNAS. 99(8): 5476-5480.

Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environ. Health Pers. 111: 568-575.

Hayes, T.B., P. Case, S. Chi, D. Chung, C. Haeffele, K. Haston, M. Lee, V.P. Mai, Y. Marjuoa, J. Parker, M. Tsui, M. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environ. Health Pers. 114(1): 40-50.

He P., W. He, A. Wang, T. Xia, B. Xu, M. Zhang, X. Chen. 2008. PBDE-47-induced oxidative stress, DNA damage and apoptosis in primary cultured rat hippocampal neurons. Neuro. Tox. 29 (1): 124-129.

Kay, J.N., P. Hannigan, and D.B. Kelley. 1999. Trophic effects of androgen: development and hormonal regulation of neuron number in a sexually dimorphic vocal motor nucleus. J. Neurobiol. 40: 375 - 385.

Kelley, D.B., 1986. Neuroeffectors for vocalization in *Xenopus laevis*: hormonal regulation of sexual dimorphism. J. Neurobiol. 17(3): 231-248.

Kelley, D. (1996). Sexual differentiation in Xenopus laevis. In: R. Tinsely and H. Kobel (Eds.), The Biology of *Xenopus*. Oxford University Press, pp. 143-176.

Kelley, D. 1997. Generating sexually differentiated songs. Cur. Opin. Neurobio. 7: 839 - 843.

Kelley, D.B. 2002. Hormonal regulation of motor output in amphibians; *Xenopus laevis* vocalizations as a model system. In: D. Pfaff, A. Arnold, A. Etgen, S. Fahrbach and R. Rubin (Eds.), Hormones, Brain and Behavior. San Diego, Academic Press. 445-468.

Kelley D.B. 1986. Neuroeffectors for vocalization in Xenopus laevis: hormonal regulation of sexual dimorphism. J. Neurobiol. 17(3): 231-248.

Kelley D.B. and J. Dennison. 1990. The vocal motor neurons of Xenopus laevis: development of sex differences in axon number. J. Neurobiol. 21(6): 869-882.

Kelley D.B., S. Fenstemaker, P. Hannigan, S. Shih. 1988. Sex differences in the motor nucleus of cranial nerve IX-X in *Xenopus laevis*: a quantitative Golgi study. J. Neurobiol. 19(5): 413-429.

Kodavanti, P.R.S. and T.R.Ward. 2005. Differential effects of commercial polybrominated diphenyl ether and polychlorinated biphenyl mixtures on intracellular signaling in rat brain *in vitro*. Tox. Sci. 85(2): 952-962.

Kuriyama, S. and I. Chahoud. 2003. Maternal exposure to low dose 2,2', 4, 4', 5 pentabromodiphenyl ether (PBDE 99) impairs male reproductive performance in adult rat offspring. Organohalogen Cpds. 1-4.

Kuriyama, S., C.E. Talsness, K. Grote, I. Chahoud. 2005. Developmental exposure to low-dose PBDE-99: effects on male fertility and neurobehavior in rat offspring. Environ. Health Pers. 113(2): 149-154.

Law, R.J., C.R. Allchin, J. de Boer, A. Covaci, D. Herzke, P. Lepom, S. Morris, J. Tronczynski, C.A. de Wit. 2006. Levels and trends of brominated flame retardants in the European environment. Chemosphere. 64: 187-208.

Legler, J. 2008. New insights into the endocrine disrupting effects of brominated flame retardants. Chemosphere, 73: 215-222.

Lilienthal, H., A. Hack, A. Roth-Harer, S.W. Grande, C.E. Talsness. 2006. Effects of developmental exposure to 2,2', 4,4', 5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. Environ. Health Pers. 114 (2): 194-201.

Main, K.M., H. Kiviranta, H.E. Virtanen, E. Sundqvist, J.T. Tuomisto, J. Tuomisto, T. Vartiainen, N.E. Skakkebaek, J. Toppari. 2007. Flame retardants in placenta and breast milk and cryptorchidism in newborn boys. Environ. Health Pers. 115(10): 1519-1526.

Moore, F.L., S.K. Boyd, D.B. Kelley. 2005. Historical perspective: hormonal regulation of behaviors in amphibians. Horm. Behav. 48: 373-383.

Oros, D.R., D. Hoover, F. Rodigari, D. Crane, J. Sericano. 2005. Levels and distribution of polybrominated diphenyl ethers in water, surface sediments, and bivalves from the San Francisco estuary. Environ. Sci. Tech. 39(1): 33-41.

Perez, J. and D. Kelley. 1996. Trophic effects of androgen: receptor expression and the survival of laryngeal motor neurons after axotomy. J. Neurosci. 16: 6625-6633.

Potter, K.A., T.O. Bose, A. Yamaguchi. 2005. Androgen-induced vocal transformation in adult female African clawed frogs. J. Neurophysiol. 94: 415-428.

Sassoon, D.A., G.E. Gray, and D.B. Kelley. 1987. Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. J. Neurosci. 7:3198-206.

Schechter, A., O. Papke, K.C. Tung, J. Joseph, K.C. Tung. 2005. Polybrominated diphenyl ethers (PBDEs) in US computers and domestic carpet vacuuming: possible sources of human exposure. J. Tox. and Environ. Health, Part A. 68(7): 501-513.

Schechter, A, S. Johnson-Welch, K.C. Tung, T.R. Harris, O. Papke, R. Robin. 2007. Polybrominated diphenyl ether (PBDE) levels in livers of US human fetuses and newborns. J. Tox. Environ. Health Part A. 70(1): 1-6.

Simpson HB, Tobias ML, Kelley DB. 1986. Origin and identification of fibers in the cranial nerve IX-X complex of Xenopus laevis: Lucifer Yellow backfills in vitro. J. Comp. Neurol. 244(4): 430-444.

Stoker, T.E., R.L. Cooper, C.S. Lambright, V.S. Wilson, J.E. Furr, L.E. Gray. 2005. *In vivo* and *in vitro* anti-androgenic effects of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture. Tox. Appl. Pharm., 207(1): 78-88.

Tobias, M. and Kelley, D.B. 1995. Sexual differentiation and endocrine regulation of the laryngeal synapse in *Xenopus laevis*, J. Neurobiol., 28, 515 - 526.

van der Ven, L.T.M., T. van de Kuli, A. Verhoef, P.E.G. Leonards, W. Slob, R.F. Canton, S. Germer, T. Hamers, T.J. Visser, S. Litens, H. Hakansson, Y. Fery, D. Schrenk, M. van den Berg, A.H. Piersma, J.G. Vos. 2007. A 28-day oral dose toxicity study enhanced to detect endocrine effects of a purified technical pentabromodiphenyl ether (pentaPBDE) mixture in Wistar rats. Toxicology. 245 (1-2): 109-122.

Viberg, H, Fredriksson, A, Jakobsson, E, Orn, U, and Eriksson, P. 2003. Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. Tox. Sci. 76: 112-120.

Viberg, H, Fredriksson, A, Eriksson, P. 2002. Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behavior, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. Toxicol. Sci. 67: 104-107.

Watson JT, Kelley DB. 1992. Testicular masculinization of vocal behavior in juvenile female Xenopus laevis reveals sensitive periods for song duration, rate, and frequency spectra. J. Comp. Physiol. [A]. 171(3): 343-350.

Wetzel DM, Haerter UL, Kelley DB. 1985. A proposed neural pathway for vocalization in South African clawed frogs, Xenopus laevis. J Comp Physiol. [A]. 157(6): 749-761.

Wetzel DM, Kelley DB. 1983. Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. Horm. Behav. 17(4): 388-404.

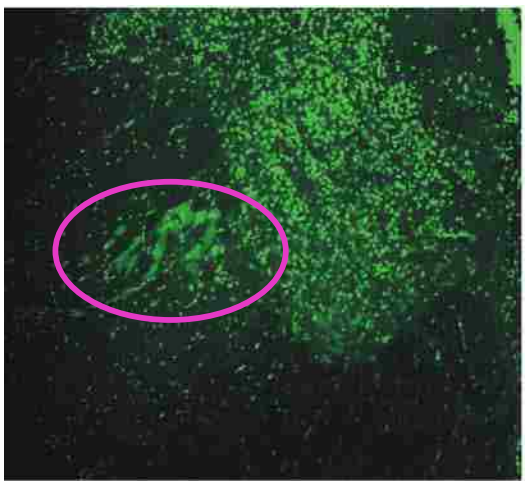
Yager, D. 1982. A novel mechanism for underwater sound production in *Xenopus borealis*. Amer. Zool. 122: 887.

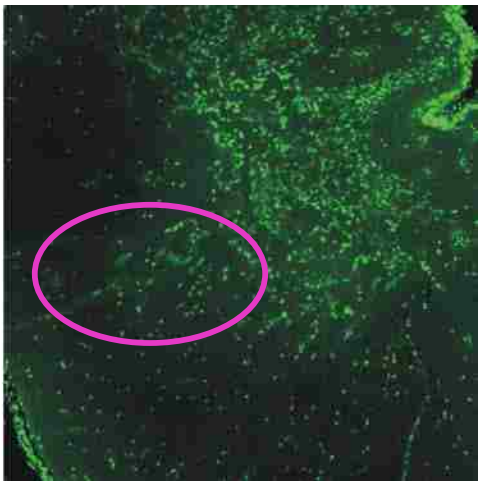
Yamaguchi, A. and Kelley, D.B. 2000. Generating sexually differentiated vocal patterns: laryngeal nerve and EMG recordings from vocalizing male and female African clawed frogs (Xenopus laevis). J. Neurosci., 20: 1559 -1567.

Zornik, E. and Kelley, D.B. 2007. Breathing and calling: neuronal networks in the Xenopus laevis hindbrain. J. Comp. Neurol., 501, 303 - 315.

APPENDIX I
CONFOCAL STUDY OF THE BRAIN: CRITICAL PERIOD TREATMENT

Low magnification (4x) rendered 100 µm image stacks through the hindbrains of newly sexually mature *X. laevis* males. Pink circles outline the cell bodies in n. IX-X. The micrograph on the left was taken from a control male, and the micrograph on the right was from a high dose PBDE-209 treated male. Differences in cell body size and number are measurable. The following micrographs are from other frogs treated with PBDE-209 during the developmental critical period. Tissues are stained with fluorescent Nissl stain (Neurotrace 488).





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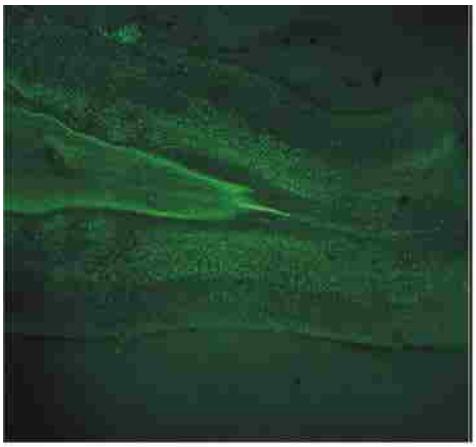

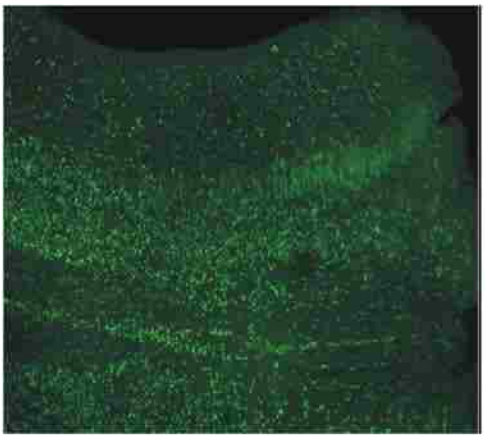

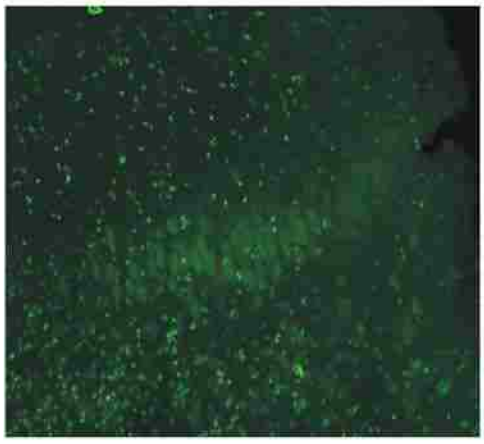

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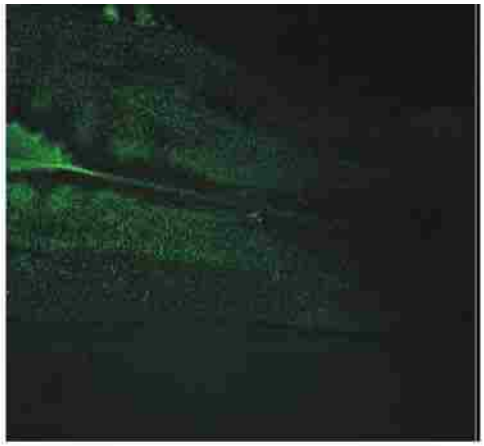

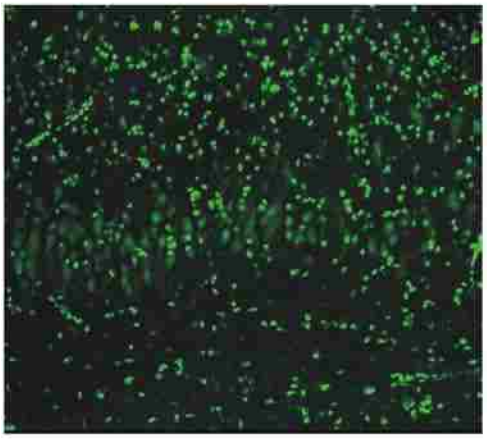

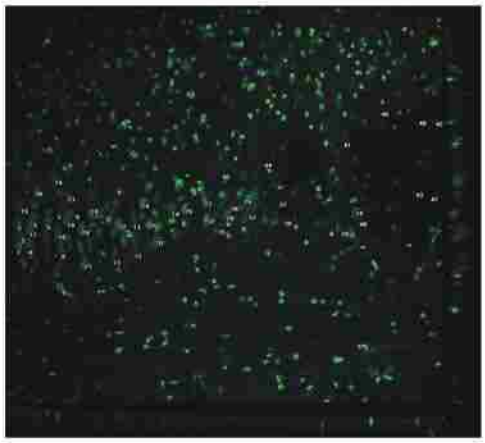

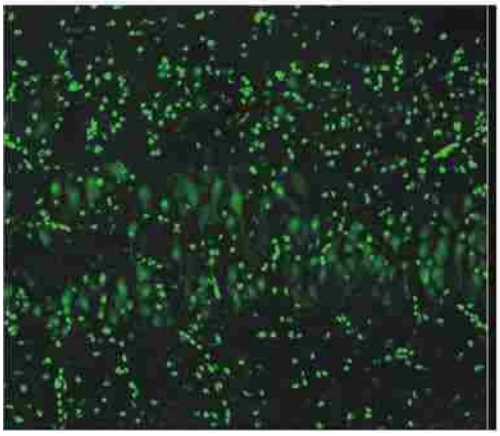

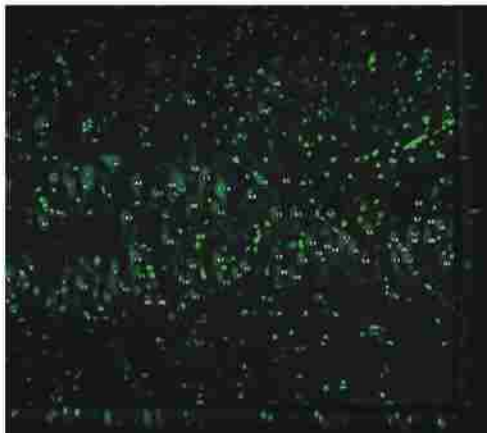

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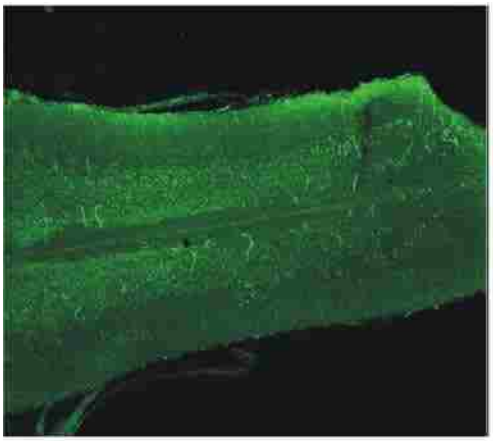

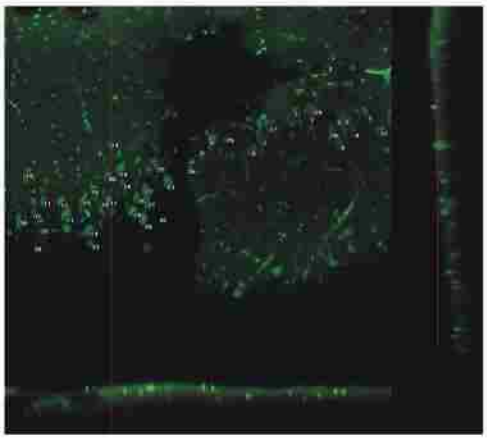

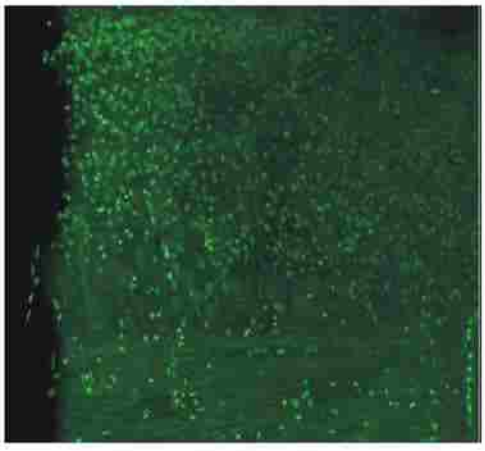

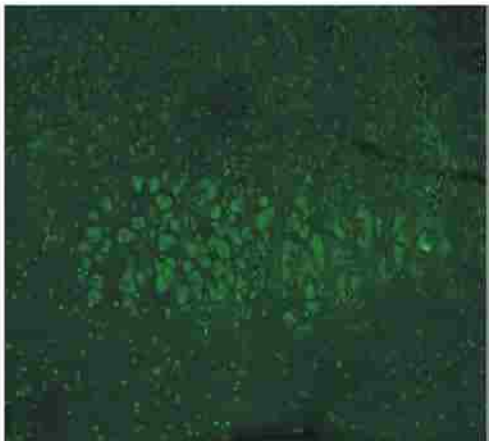

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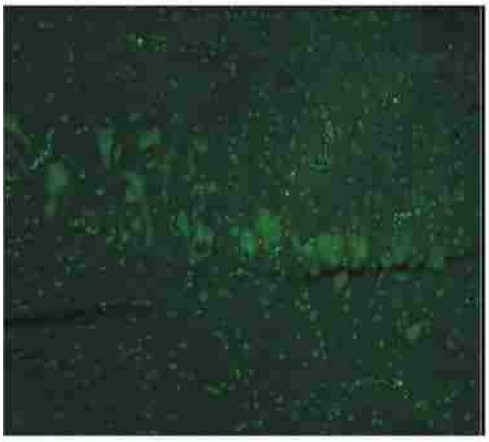

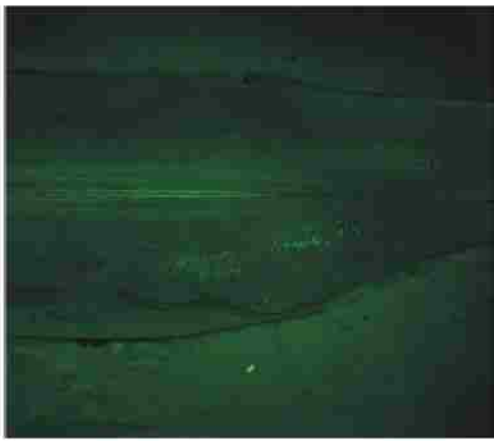

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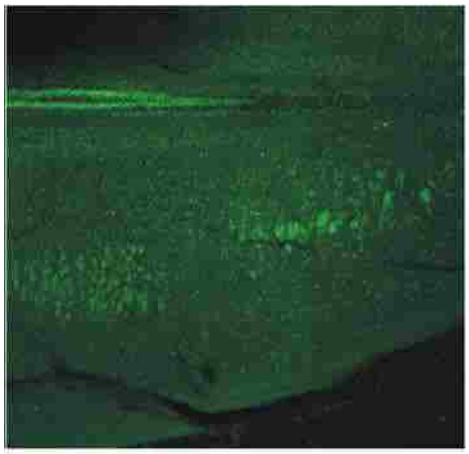

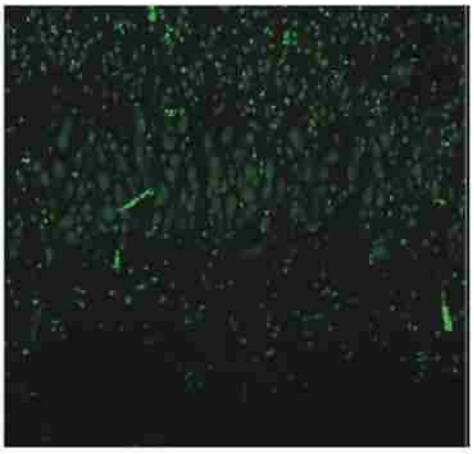

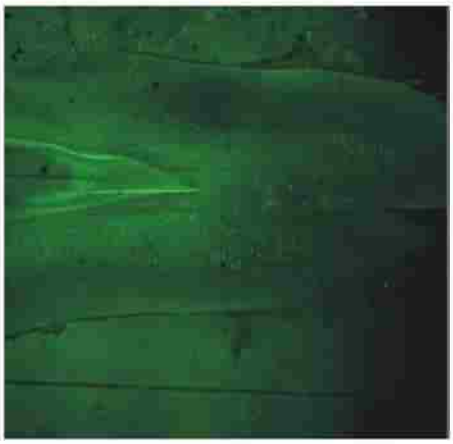

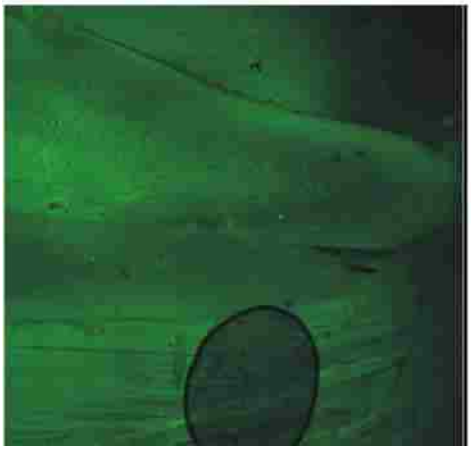

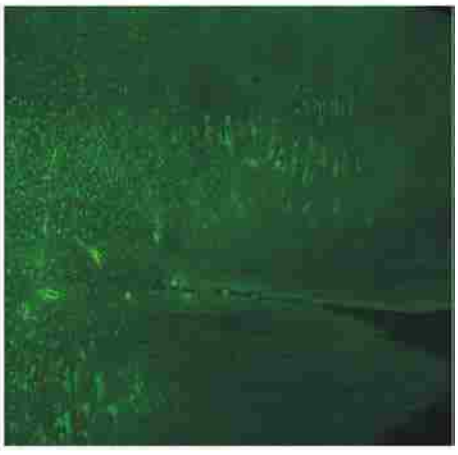

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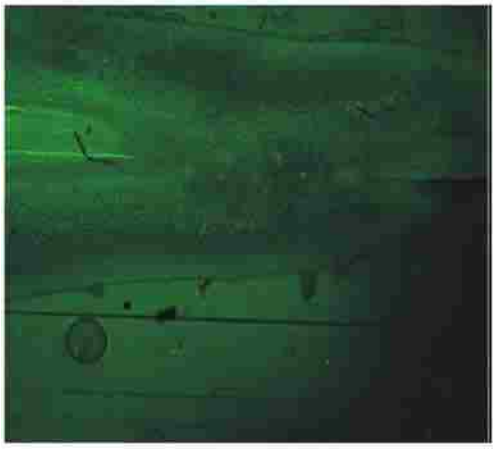
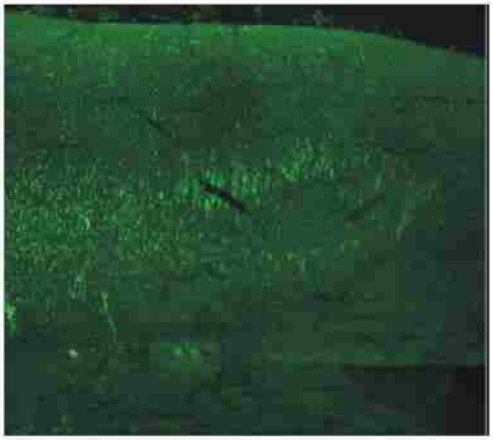
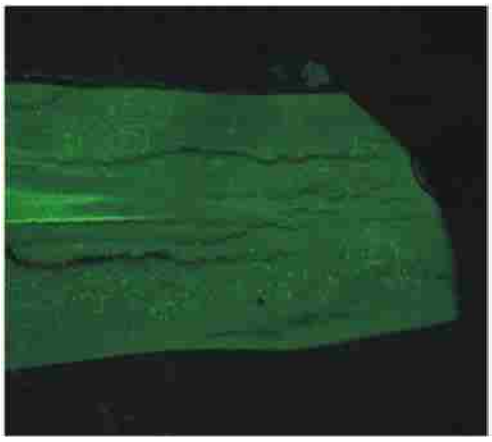
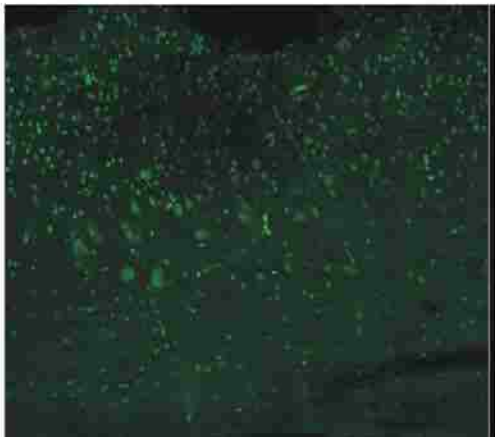

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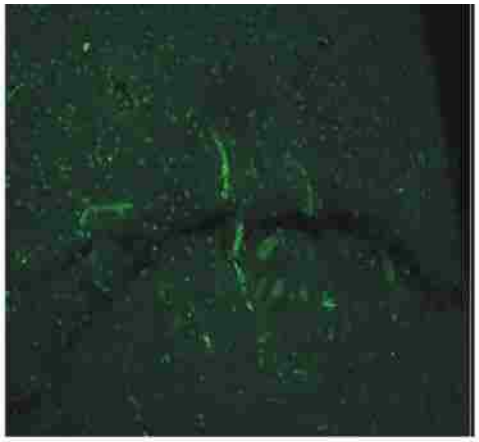

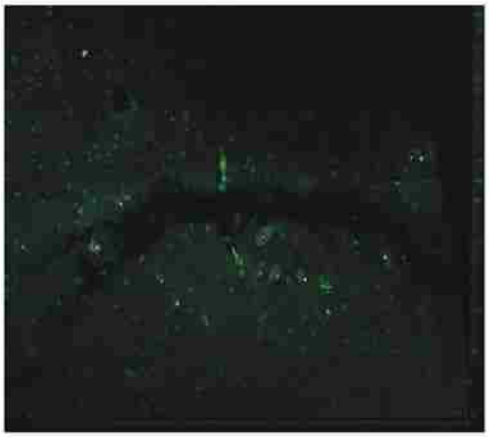

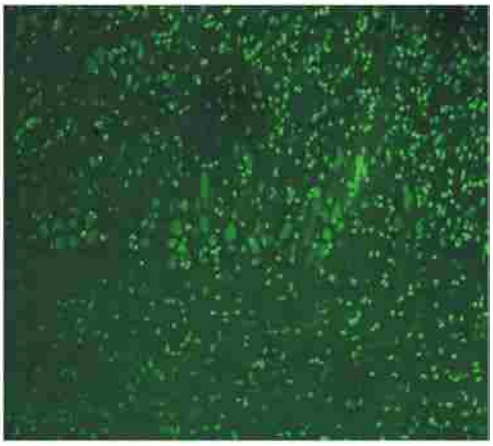

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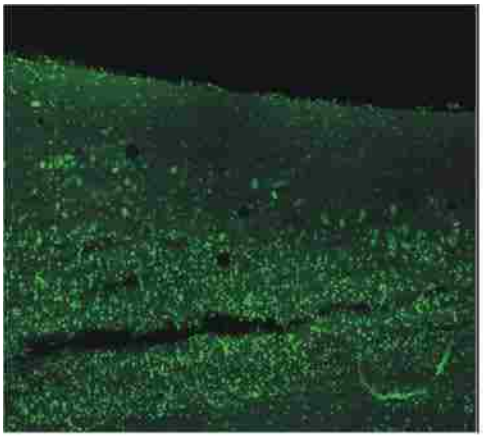
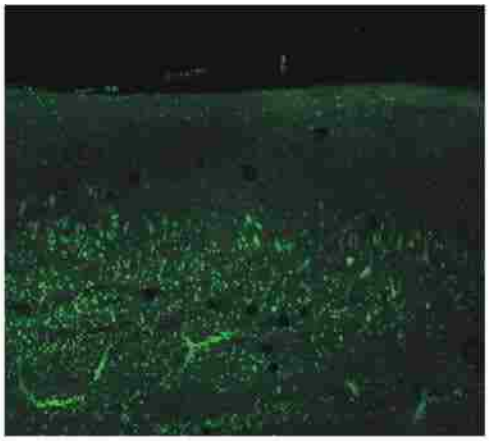
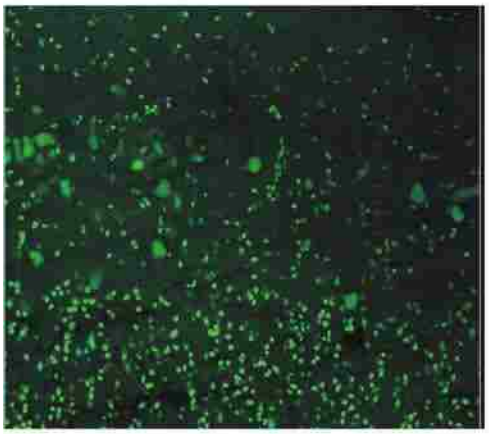
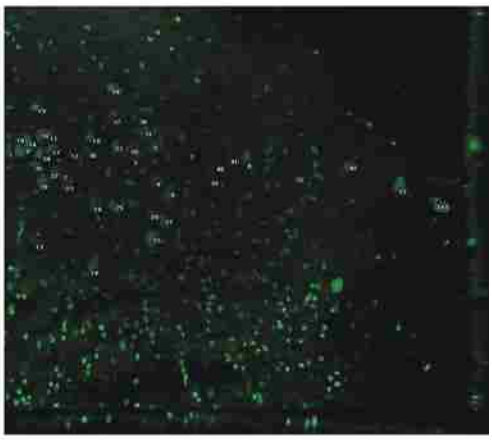
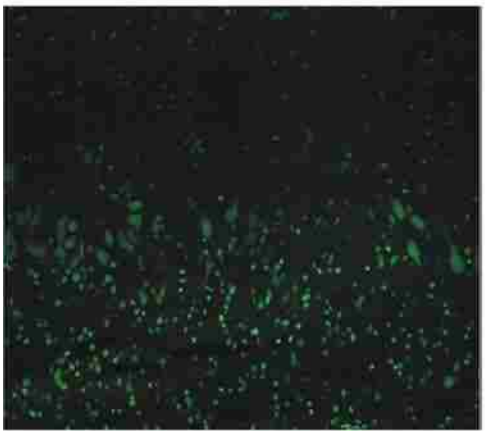


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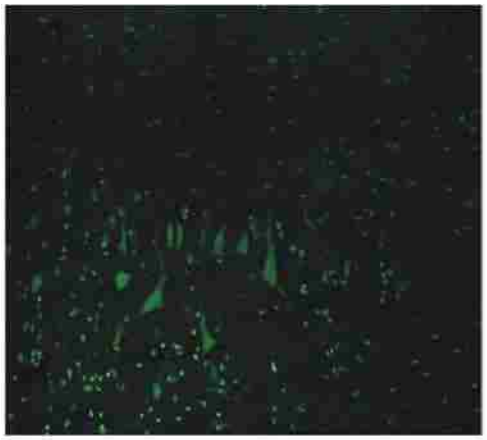

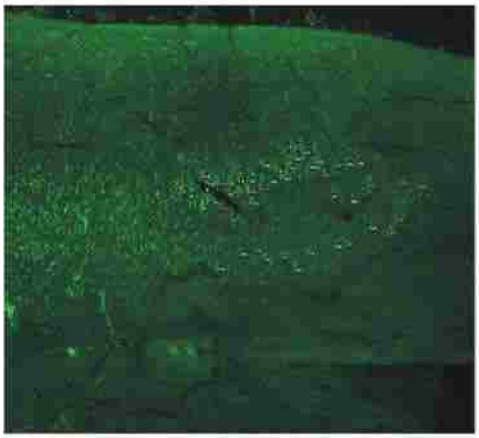
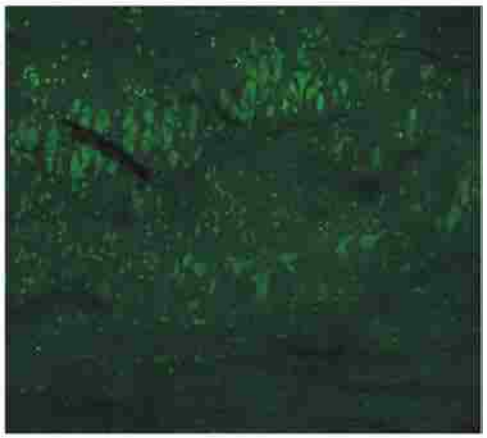
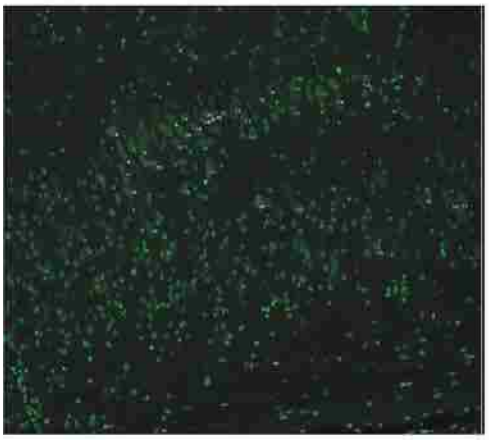
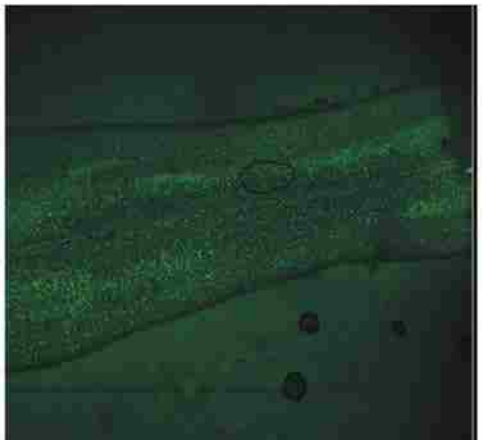

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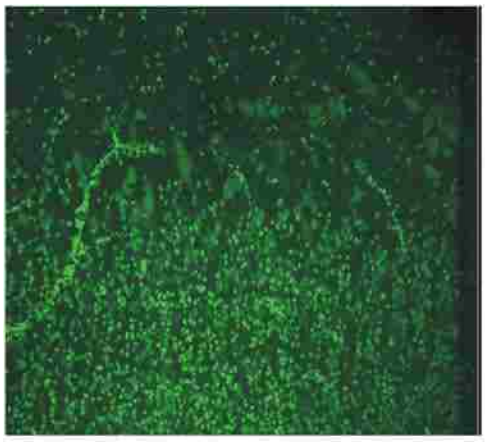
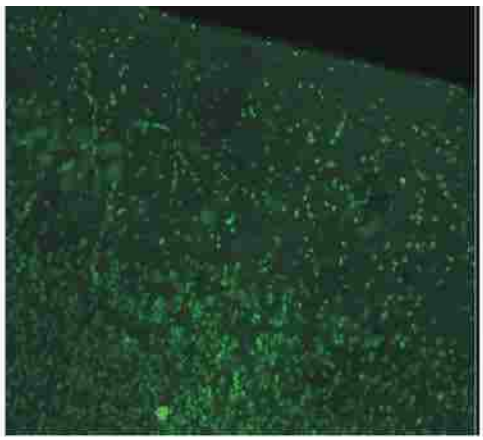
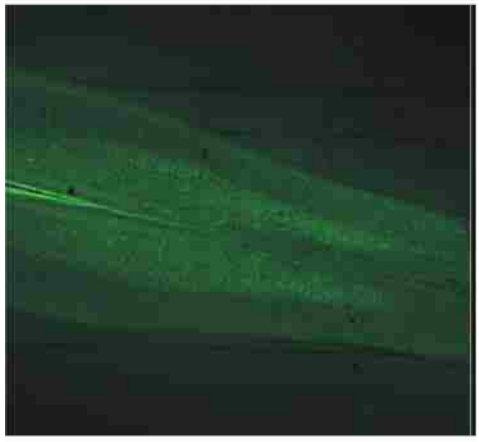
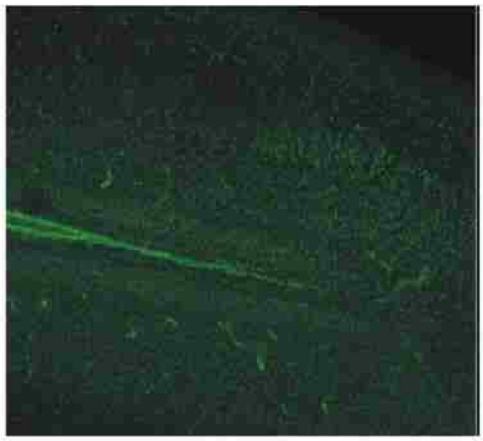
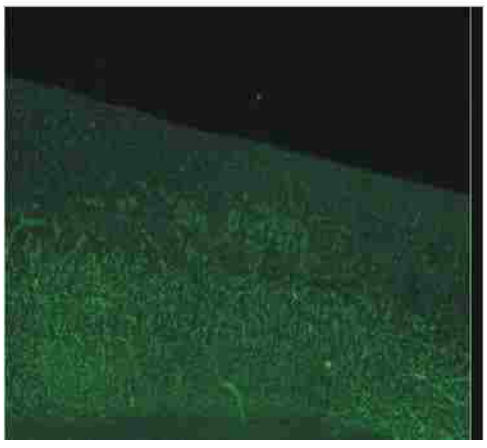


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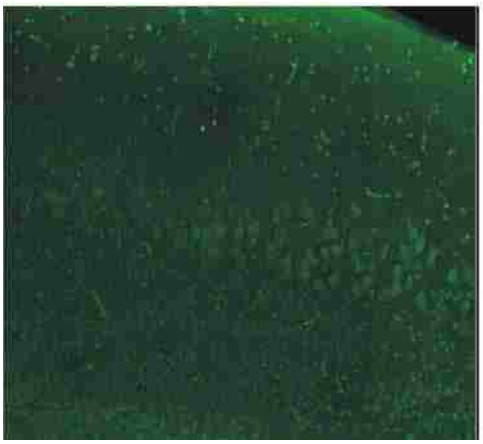

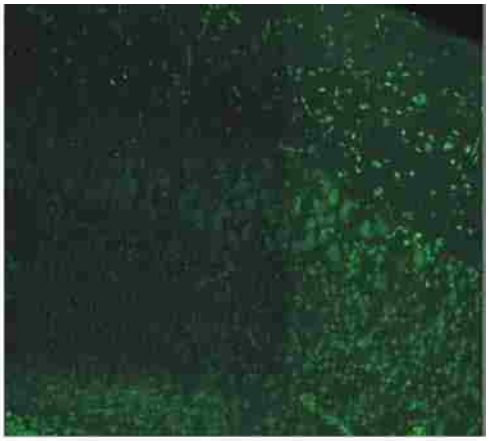

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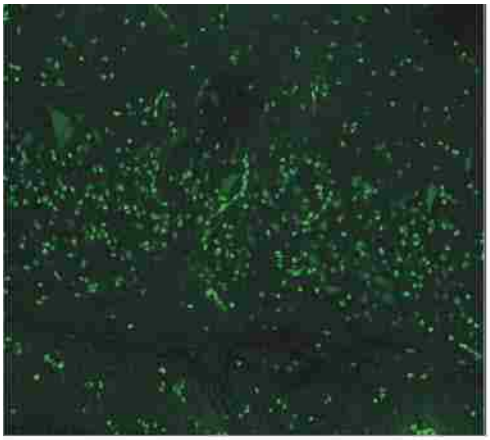

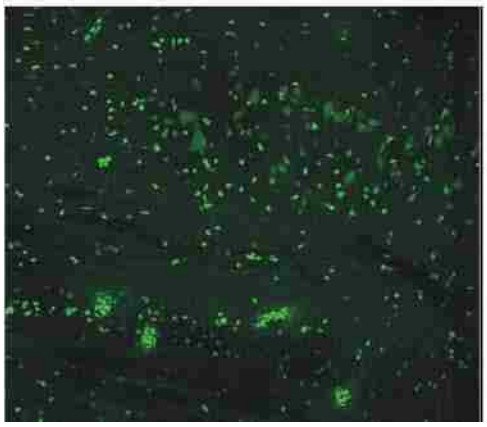

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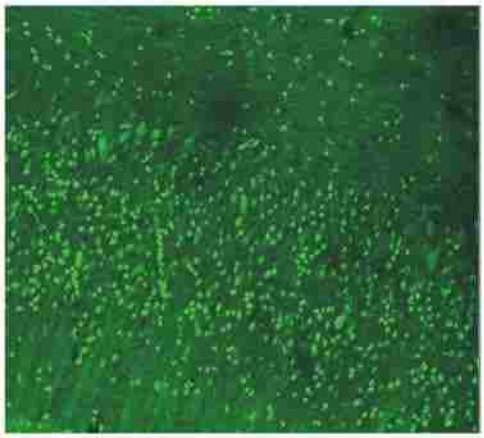
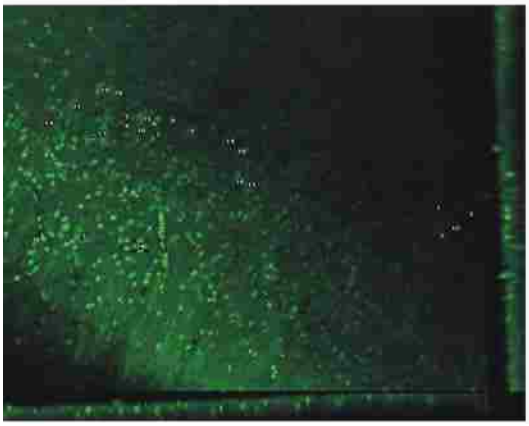
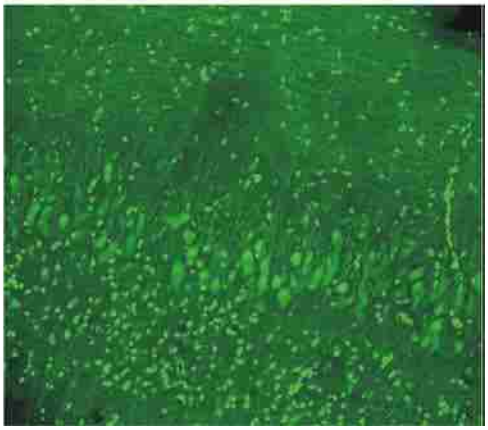
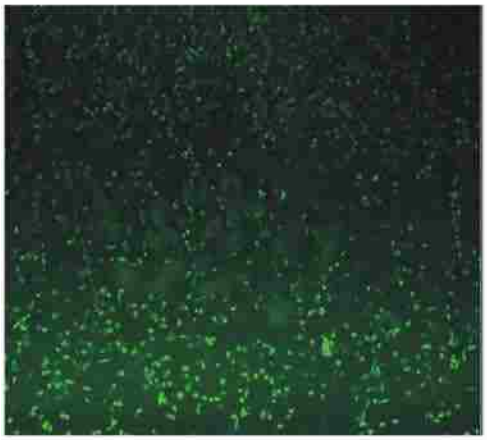
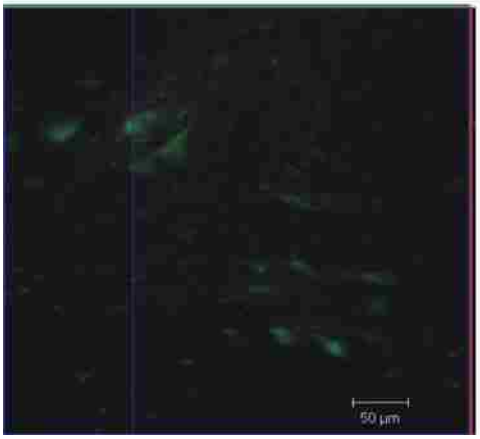






Middle Dose:





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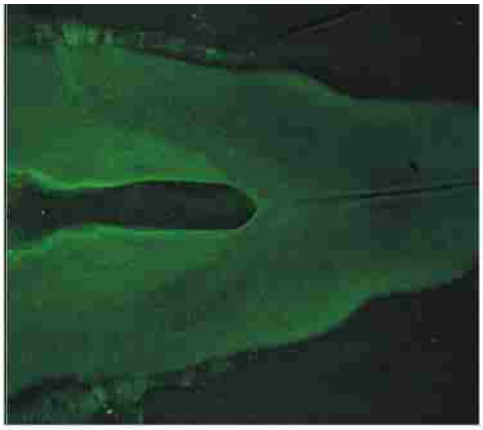

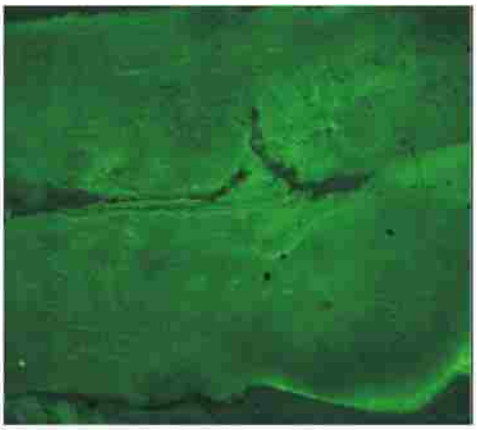

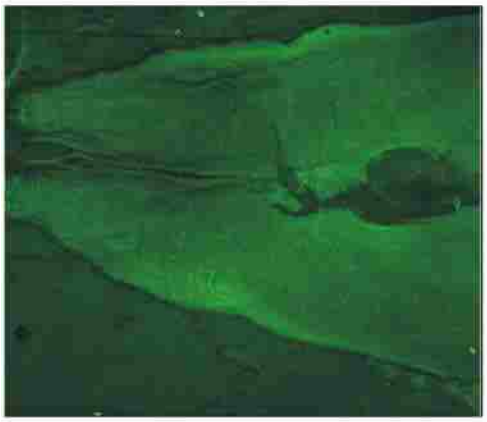

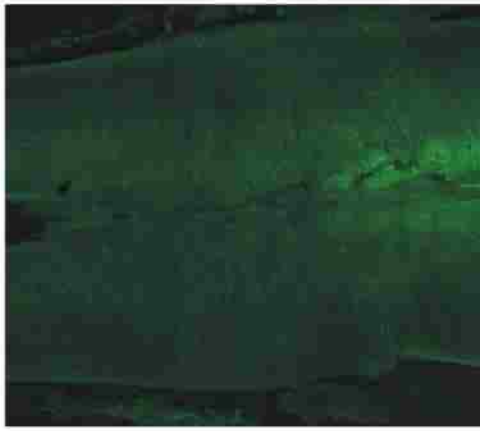

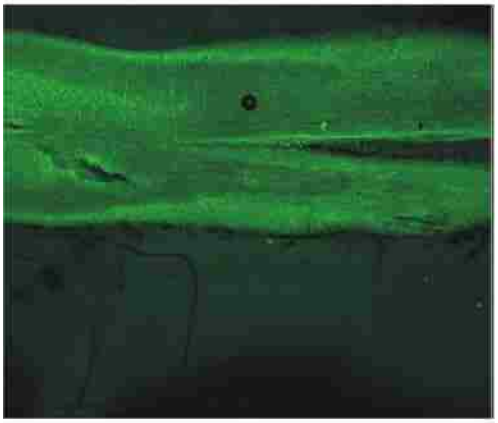

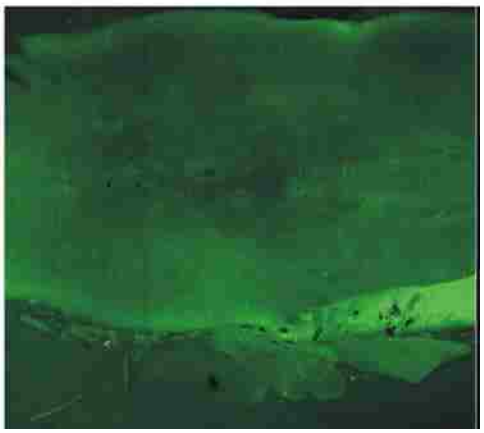

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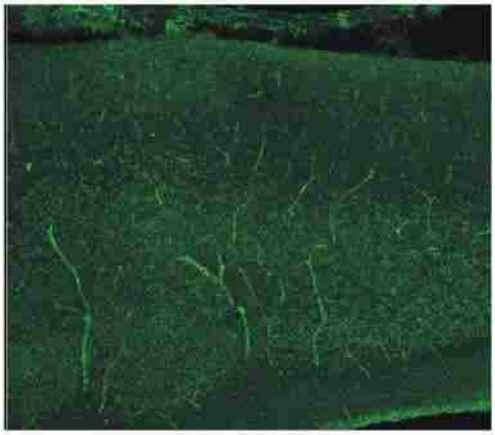

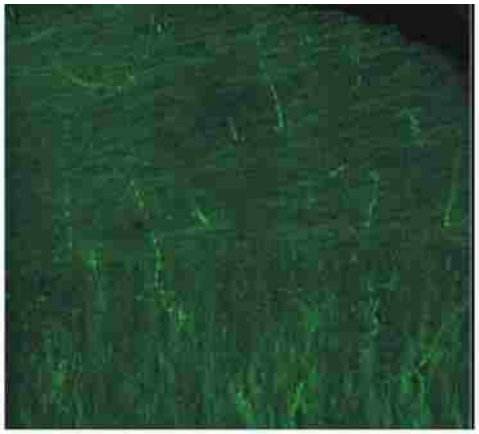

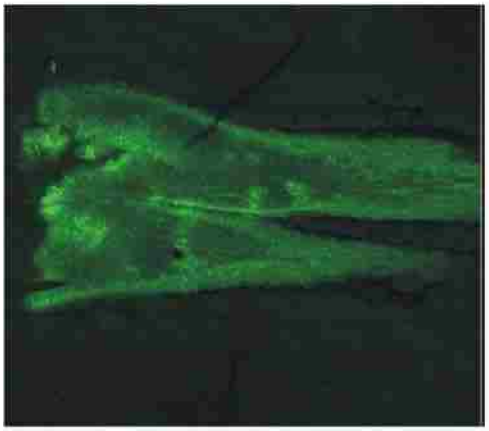

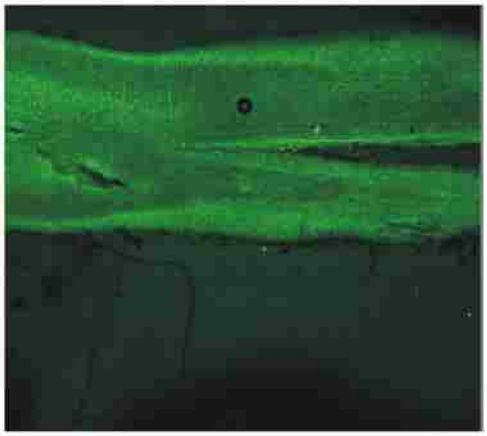

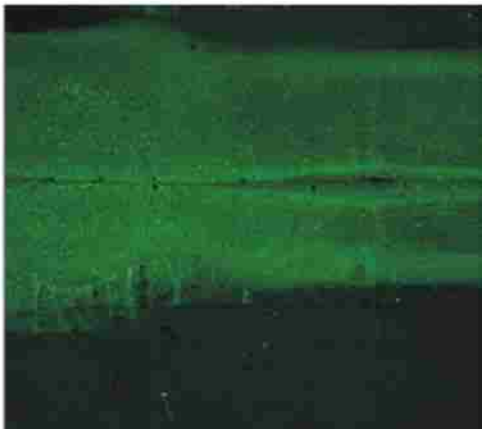

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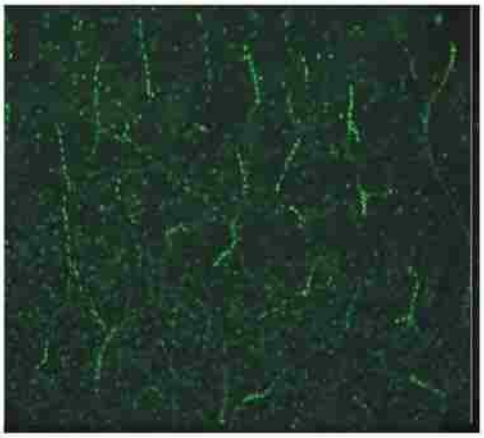
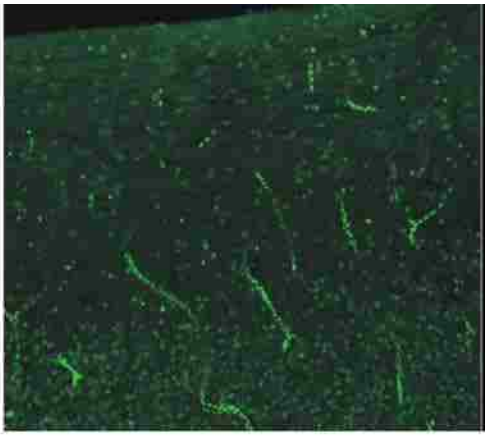
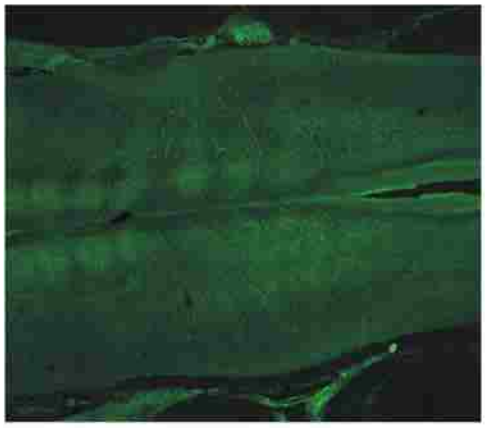
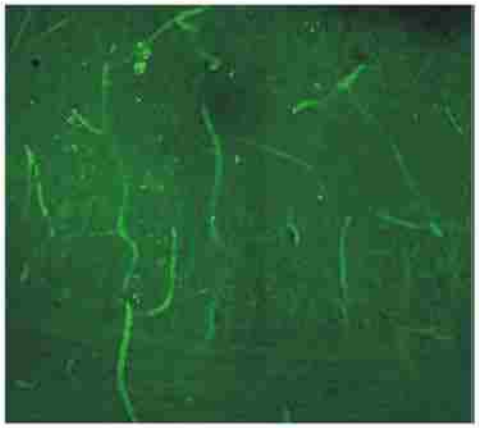
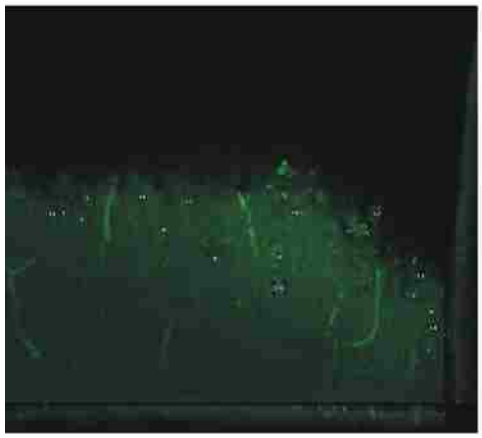
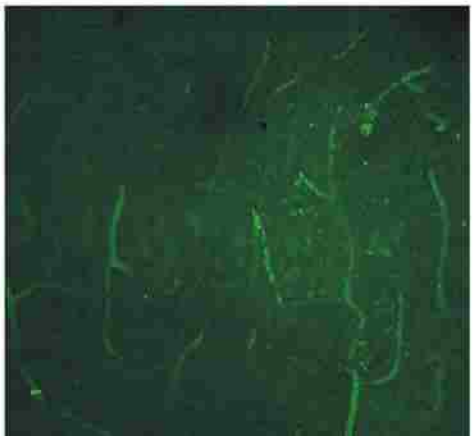






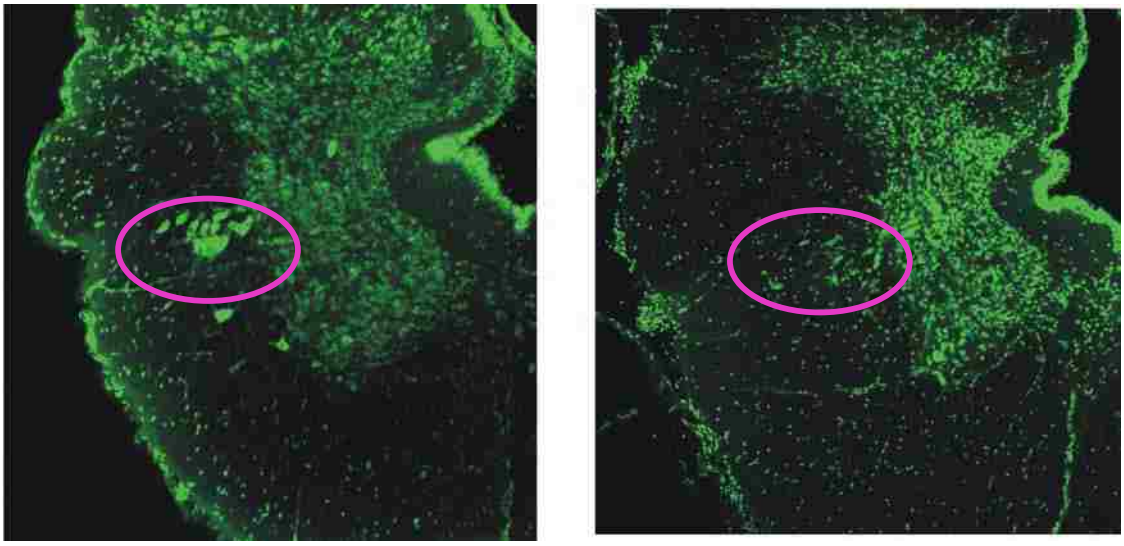




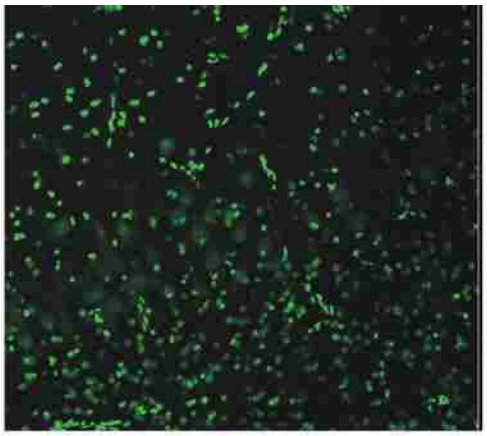
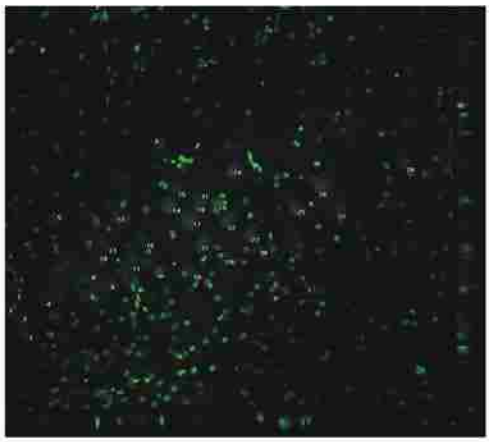
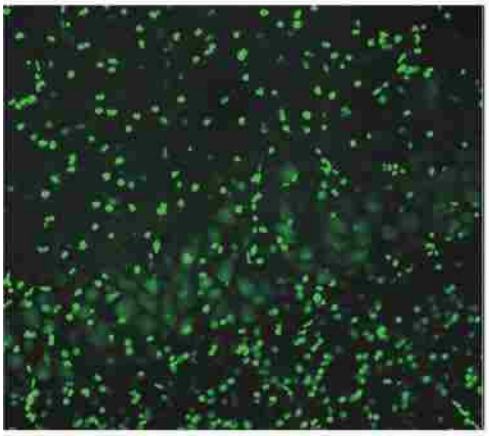
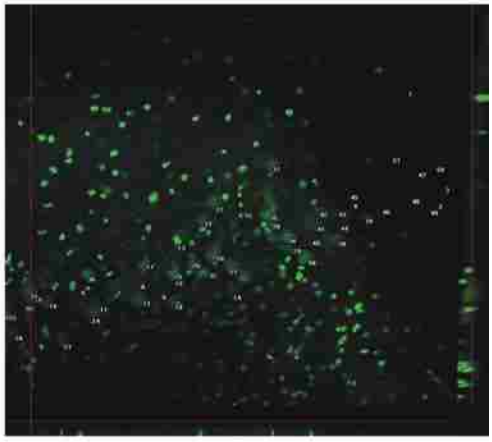
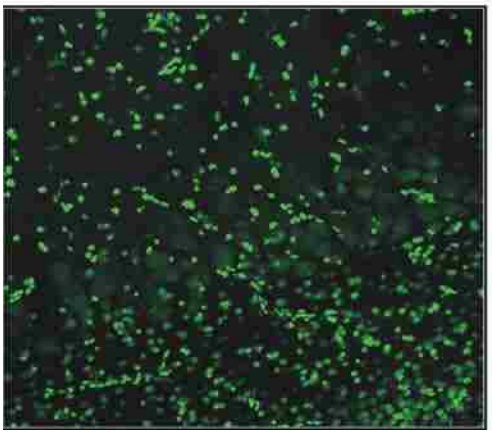
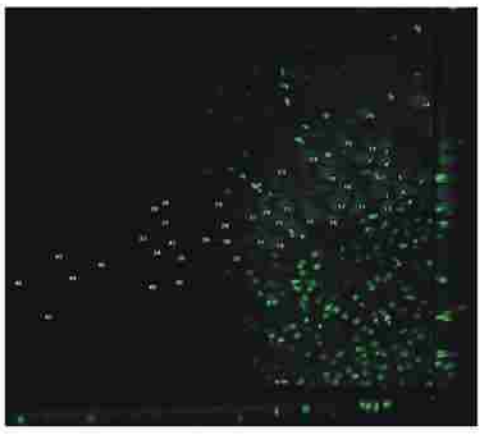


APPENDIX II
CONFOCAL STUDY OF THE ADULT BRAIN

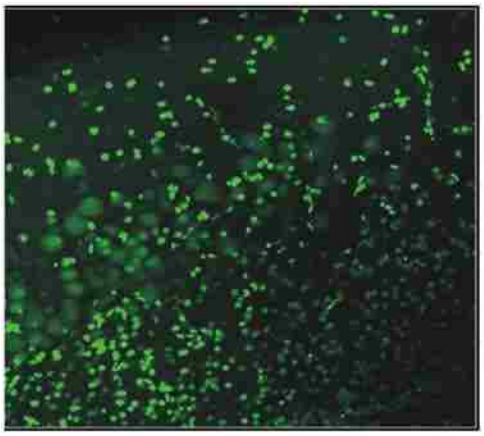
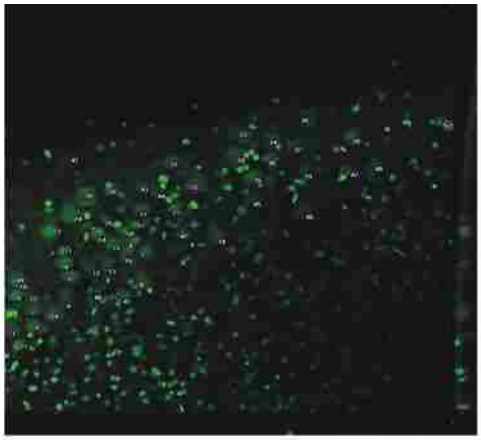
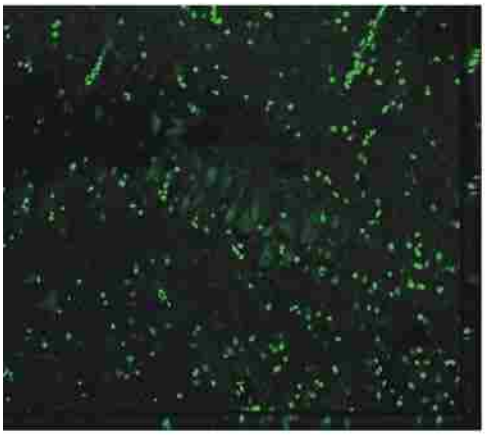
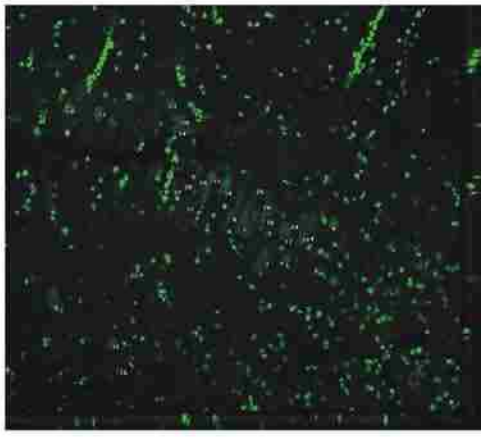
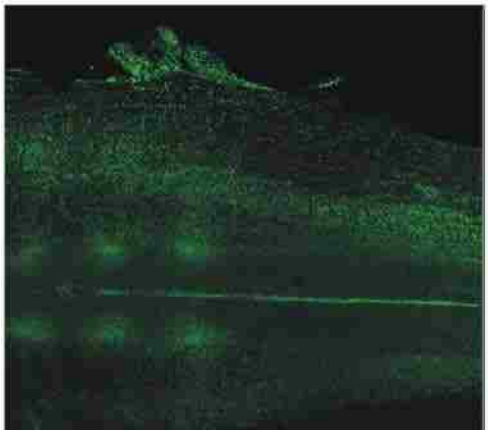
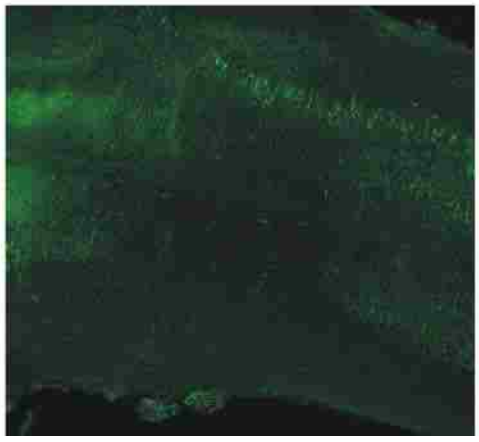
The images presented in Appendix II are representative confocal micrographs from n. IX-X containing region of the hindbrain in PBDE-209 treated adult male *X. laevis*. Tissues were labeled with the fluorescent Nissl stain, Neurotrace 488. Both low magnification and high magnification micrographs of the hindbrains are presented. The above two images represent frontal sections taken through the vocal motor nucleus (encircled in pink) in a control individual (right) and a high dose individual (left). Following the above images, the remaining tissues were sectioned along the horizontal plane and are representative renderings of 100 µm image stacks.



Control Adult:

Control Adult:

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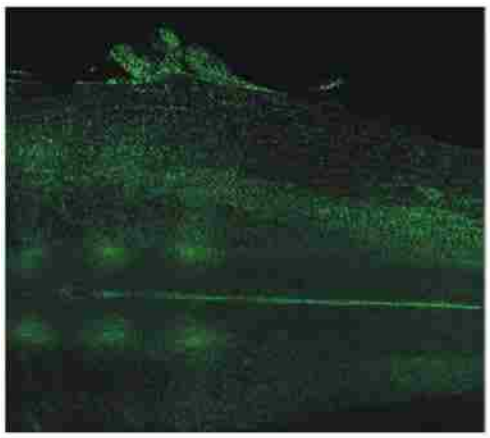
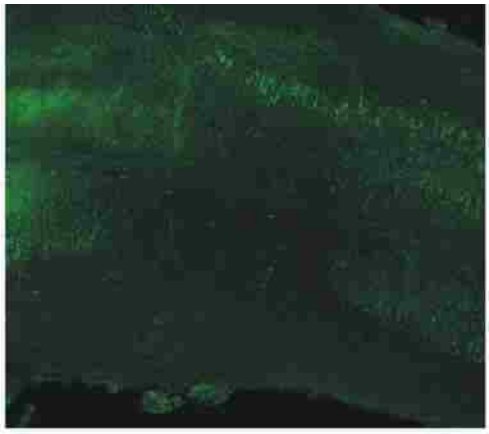
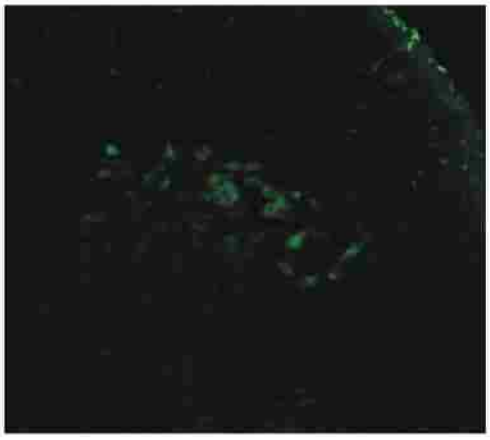
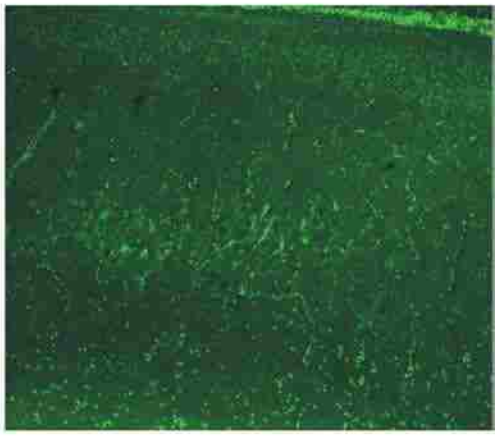
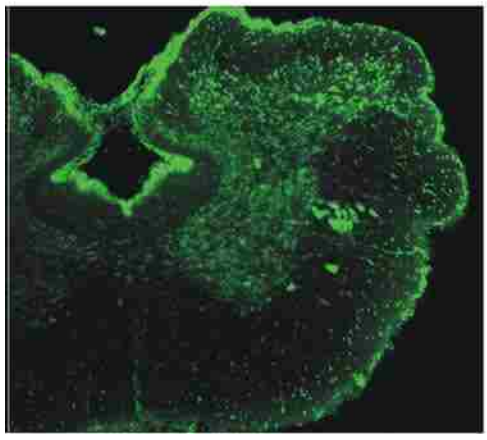
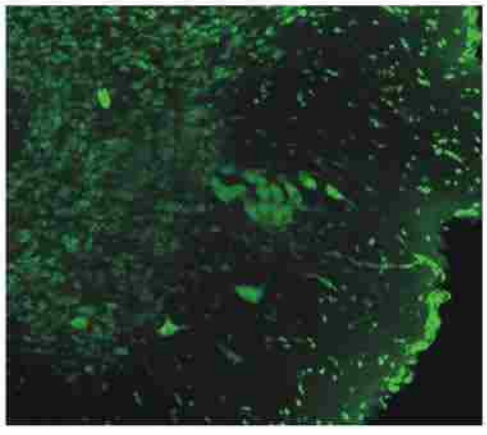

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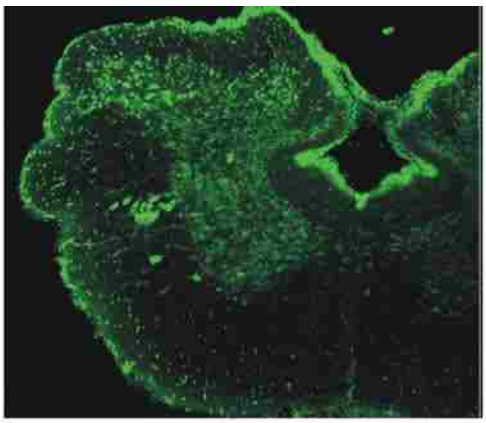
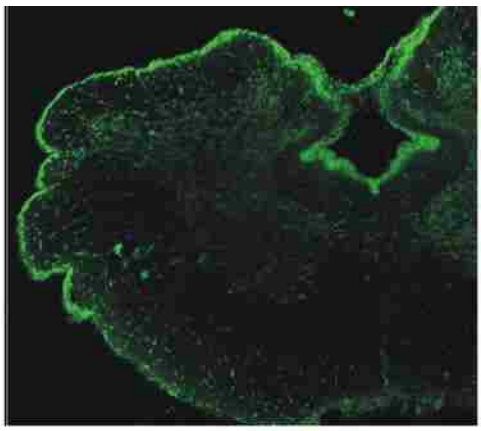
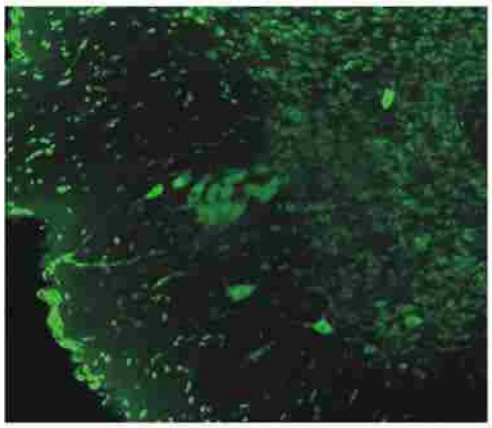
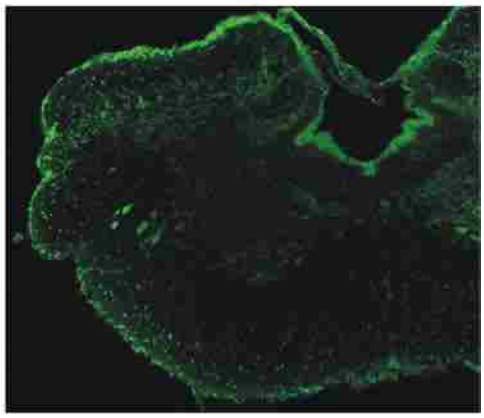
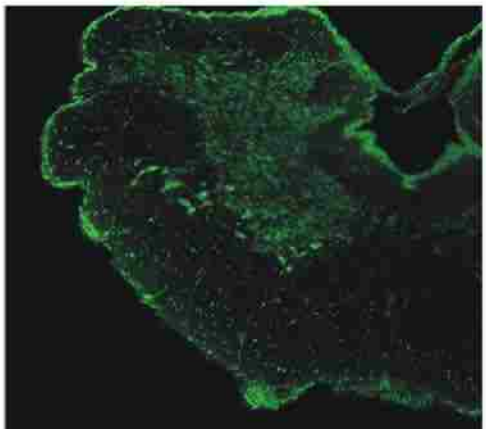
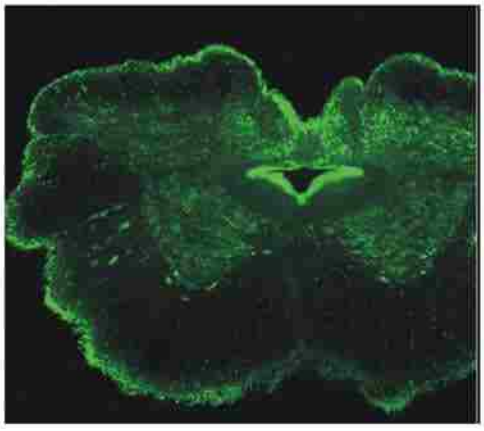

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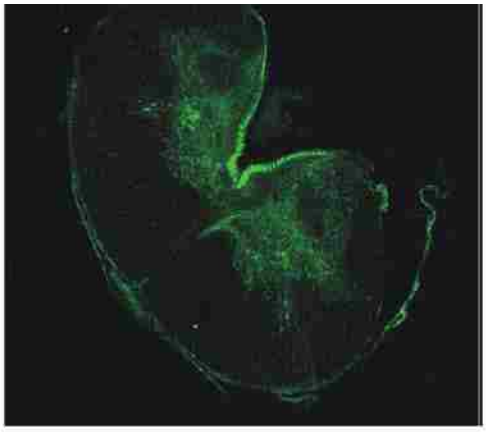

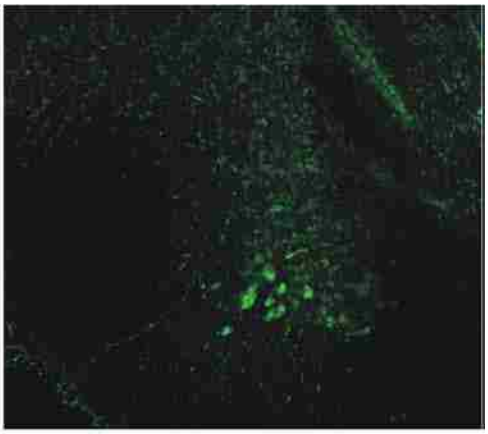

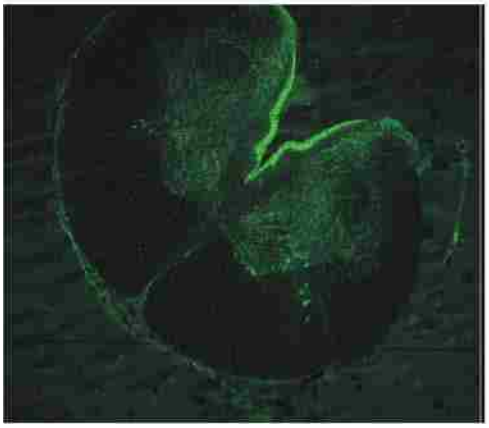

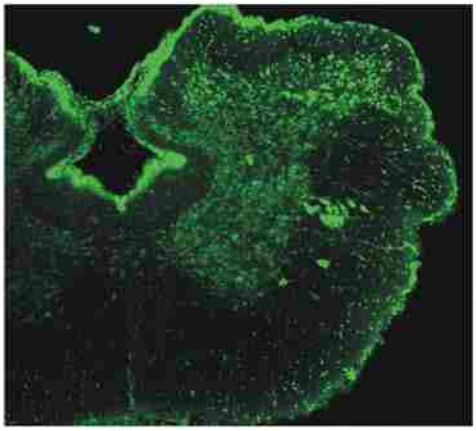

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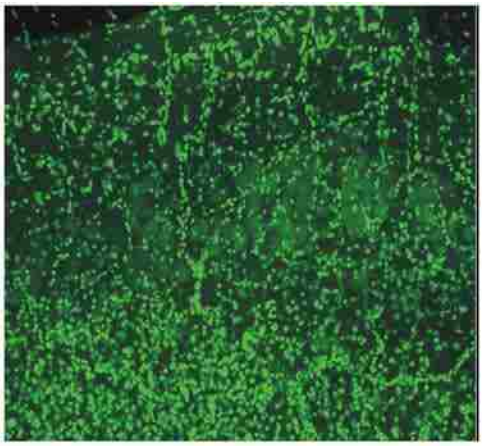

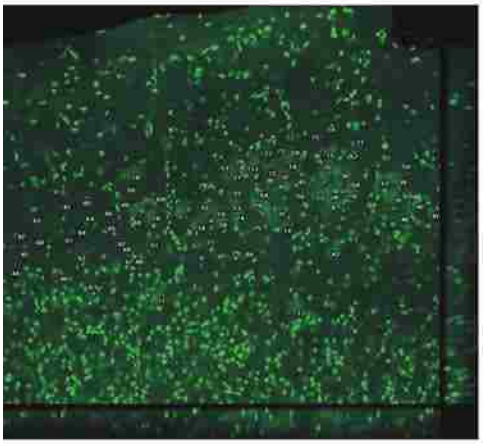

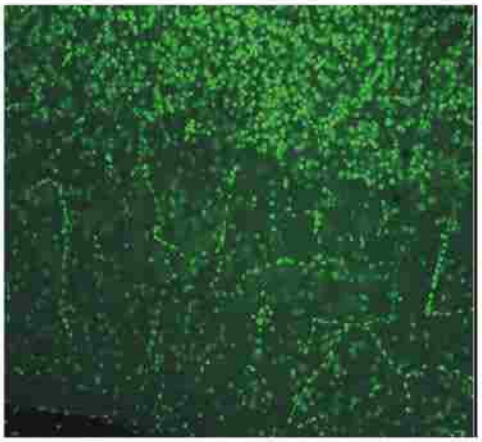

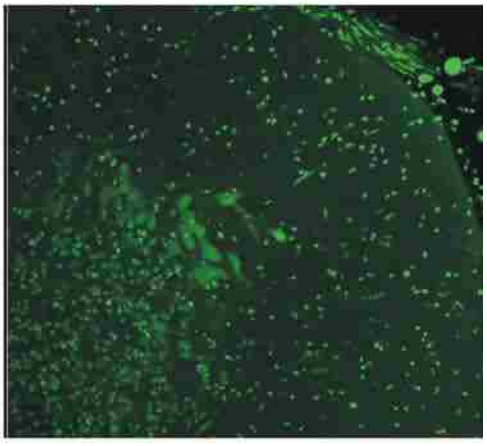

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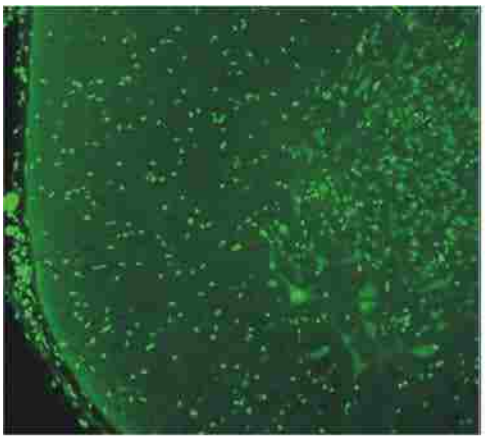
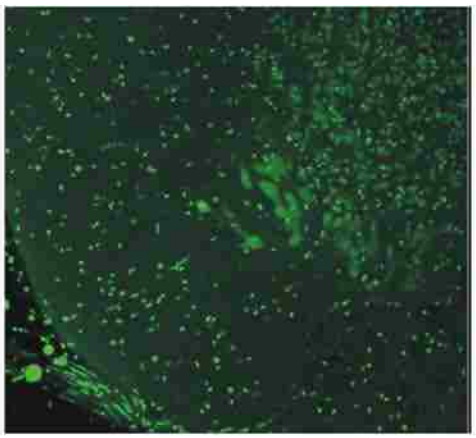
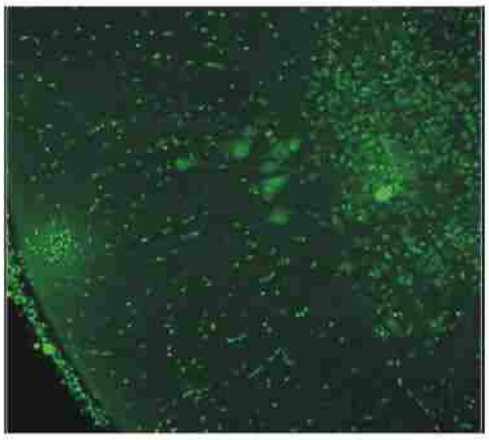
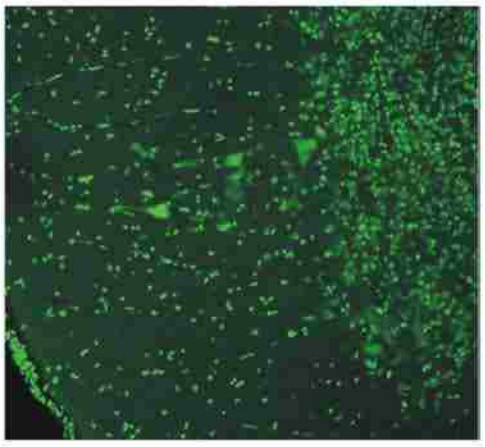
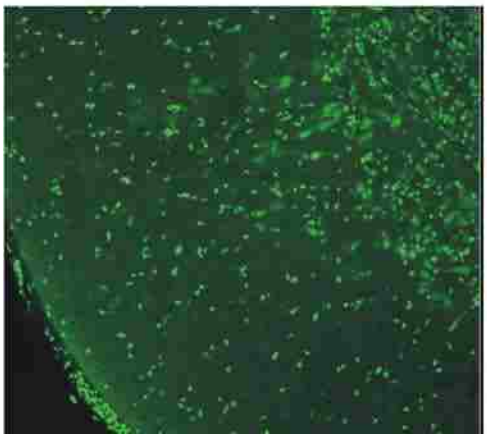
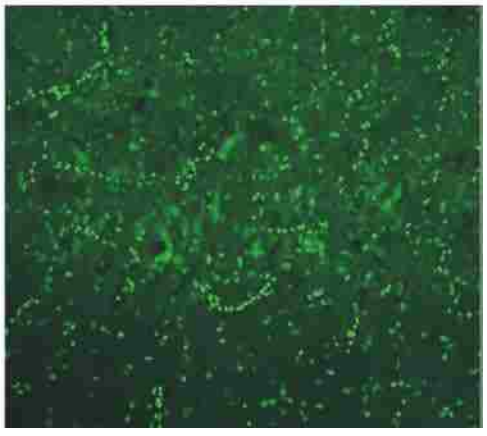

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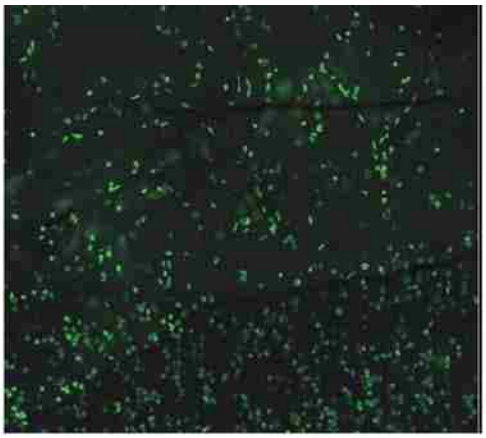

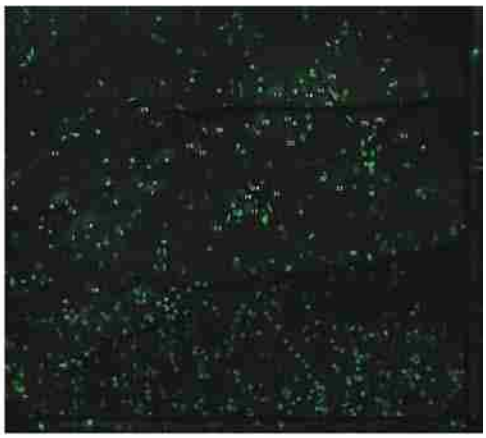

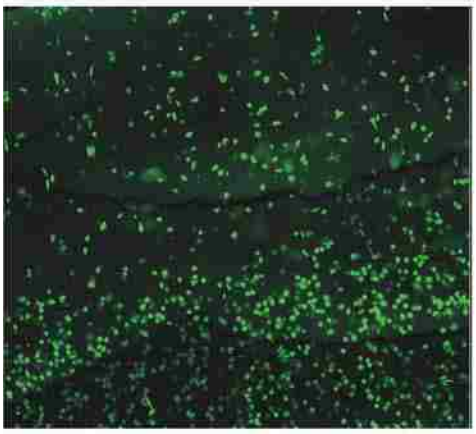

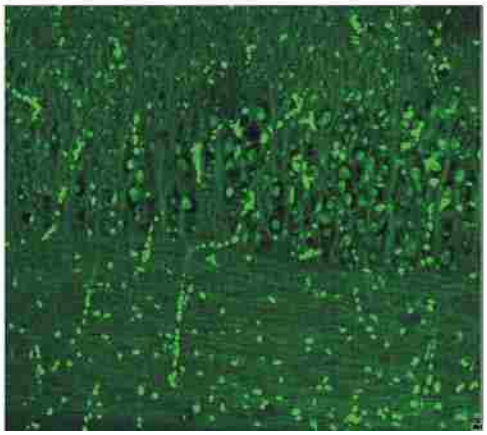

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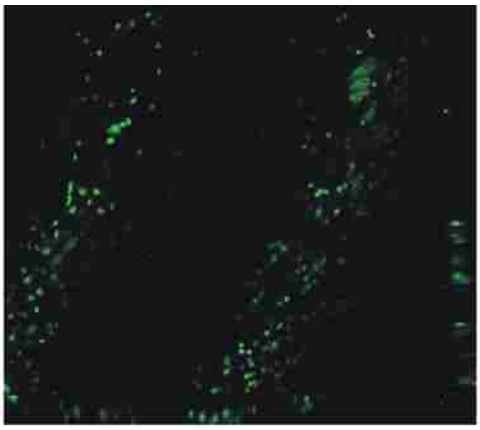

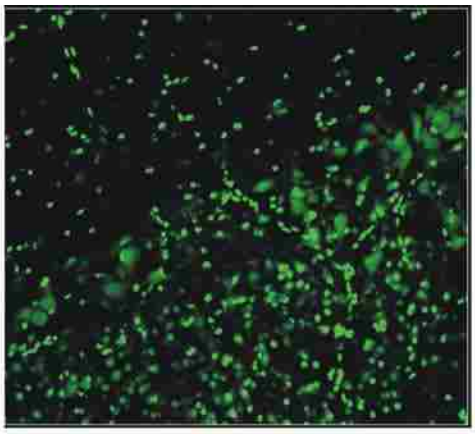

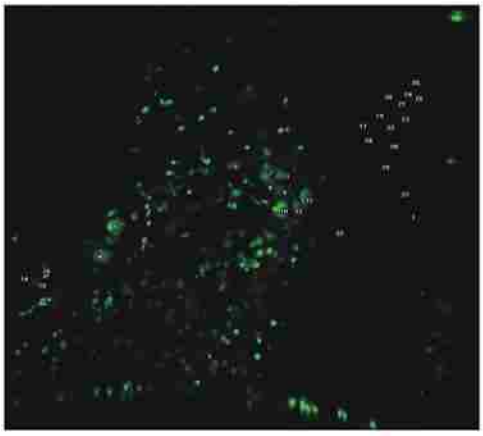

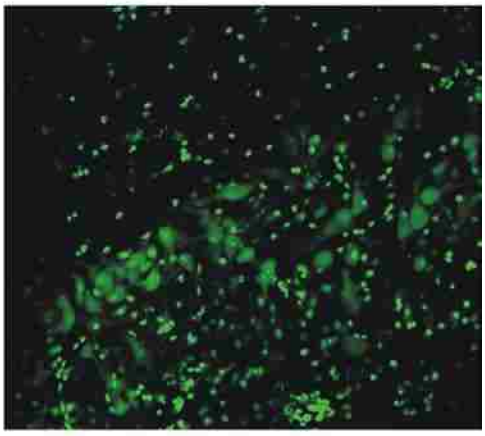

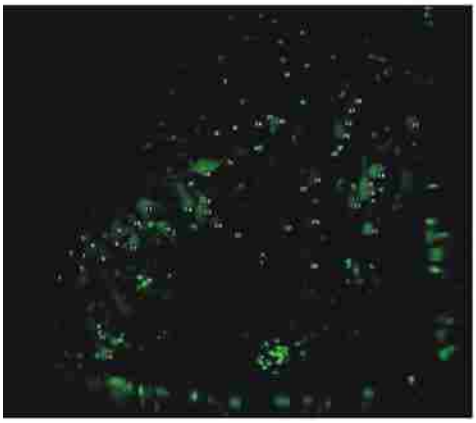

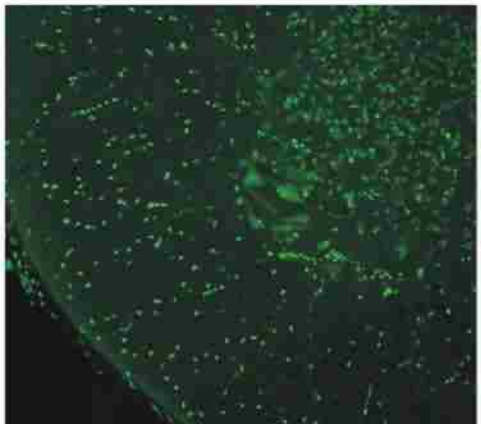

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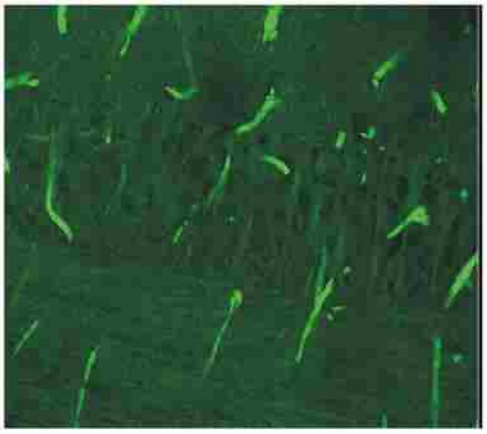
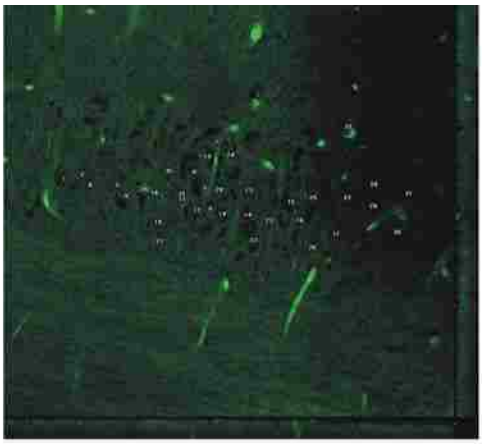

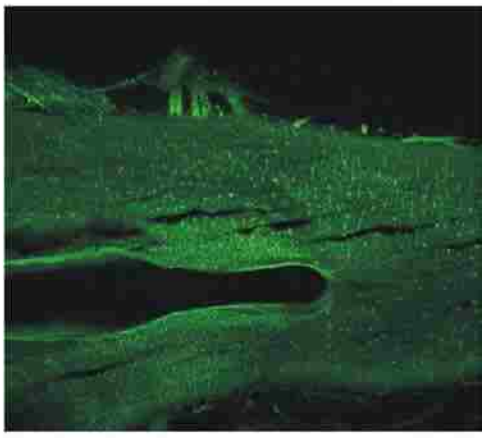
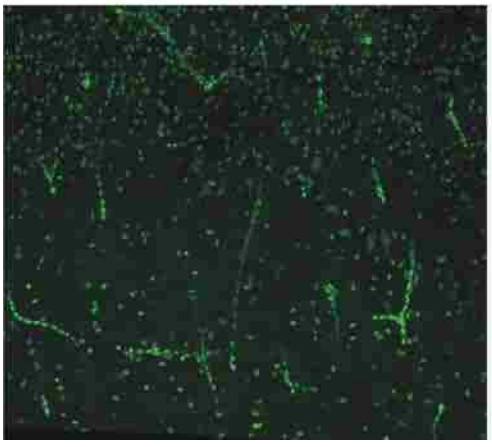
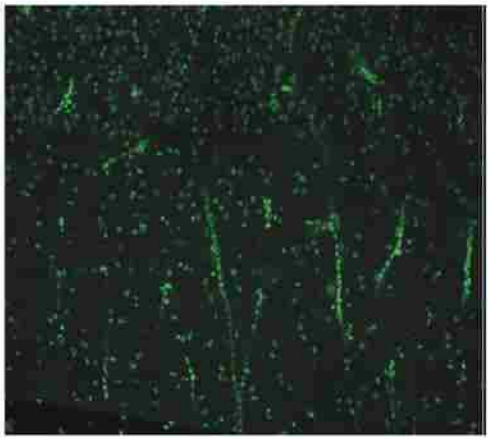

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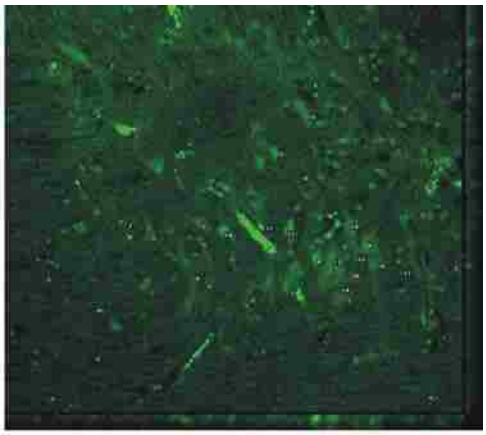
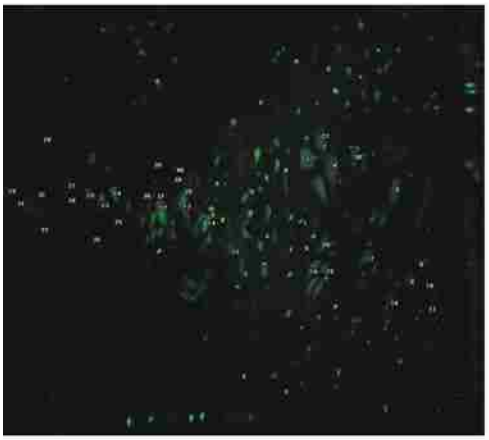
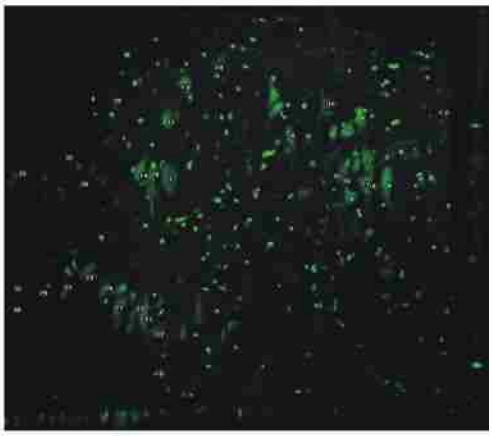
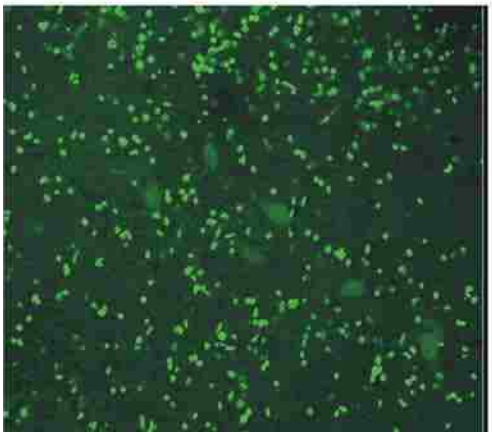
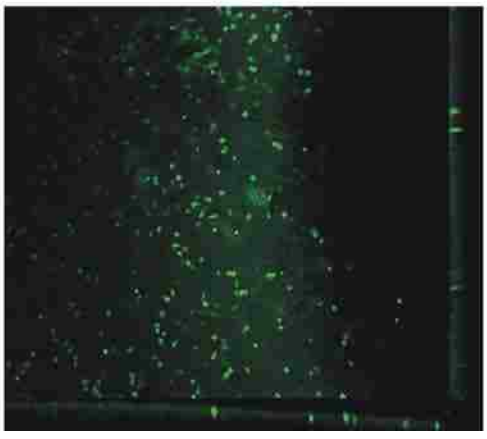
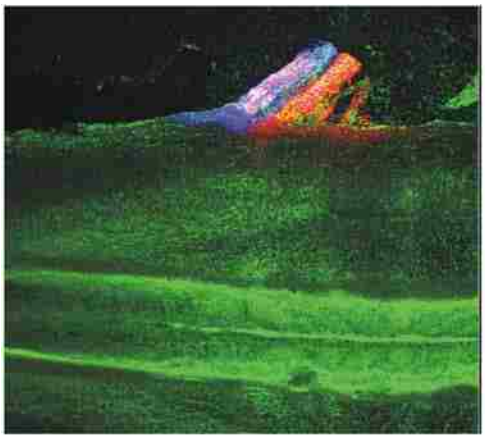






APPENDIX III
ALTERNATIVE STAINING STRATEGIES

In order to accurately determine the size of n. IX-X and its location in the hindbrain, several histological methods were employed. Horizontal sections through the hindbrain along with fluorescent Nissl staining and confocal tissue analysis were ideal and reliable methods for measuring differences in n. IX-X. Other staining and sectioning methods were also used. Retrograde lipophilic dyes, DiI (red) and DiD (blue) were injected into the fourth root (larygeal dilator nerve) or the glossopharyngeal-vagal nerve complex (N. IX-X). After six months' incubation, the hindbrain was sectioned to reveal labeling of n. IX-X somata. Three-dimensional reconstructions of the hindbrain were also made to accurately map the location of n. IX-X in the hindbrain as well as to estimate the size of n. IX-X. Serial 10 μm sections through the hindbrain were also made in order to reconstruct the hindbrain but also to accurately map the location of n. IX-X.

Retrograde Labeling DiI and DiD:



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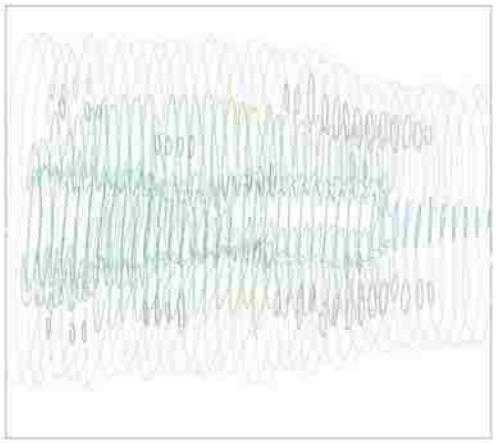
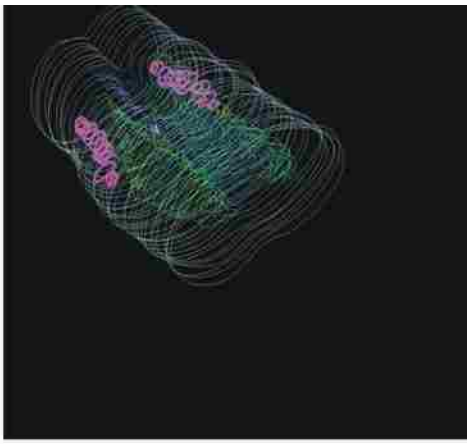
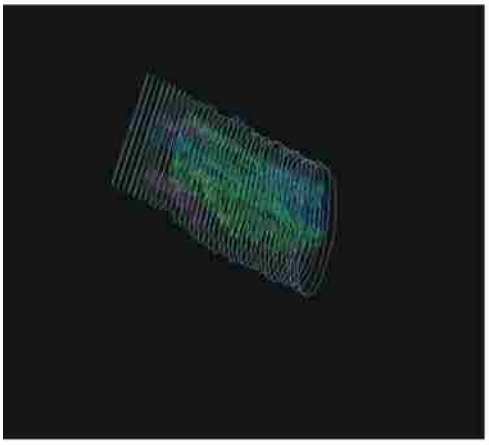
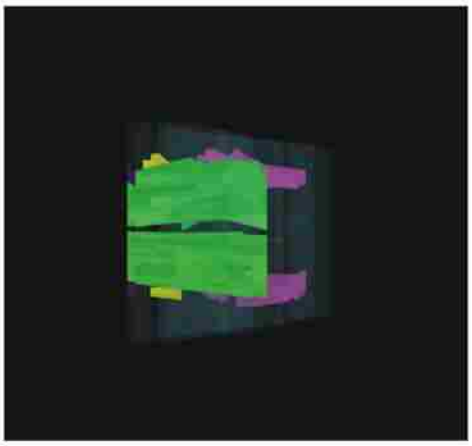
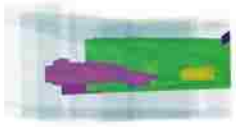
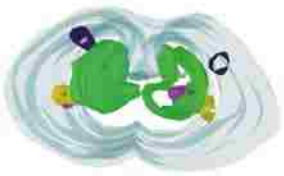




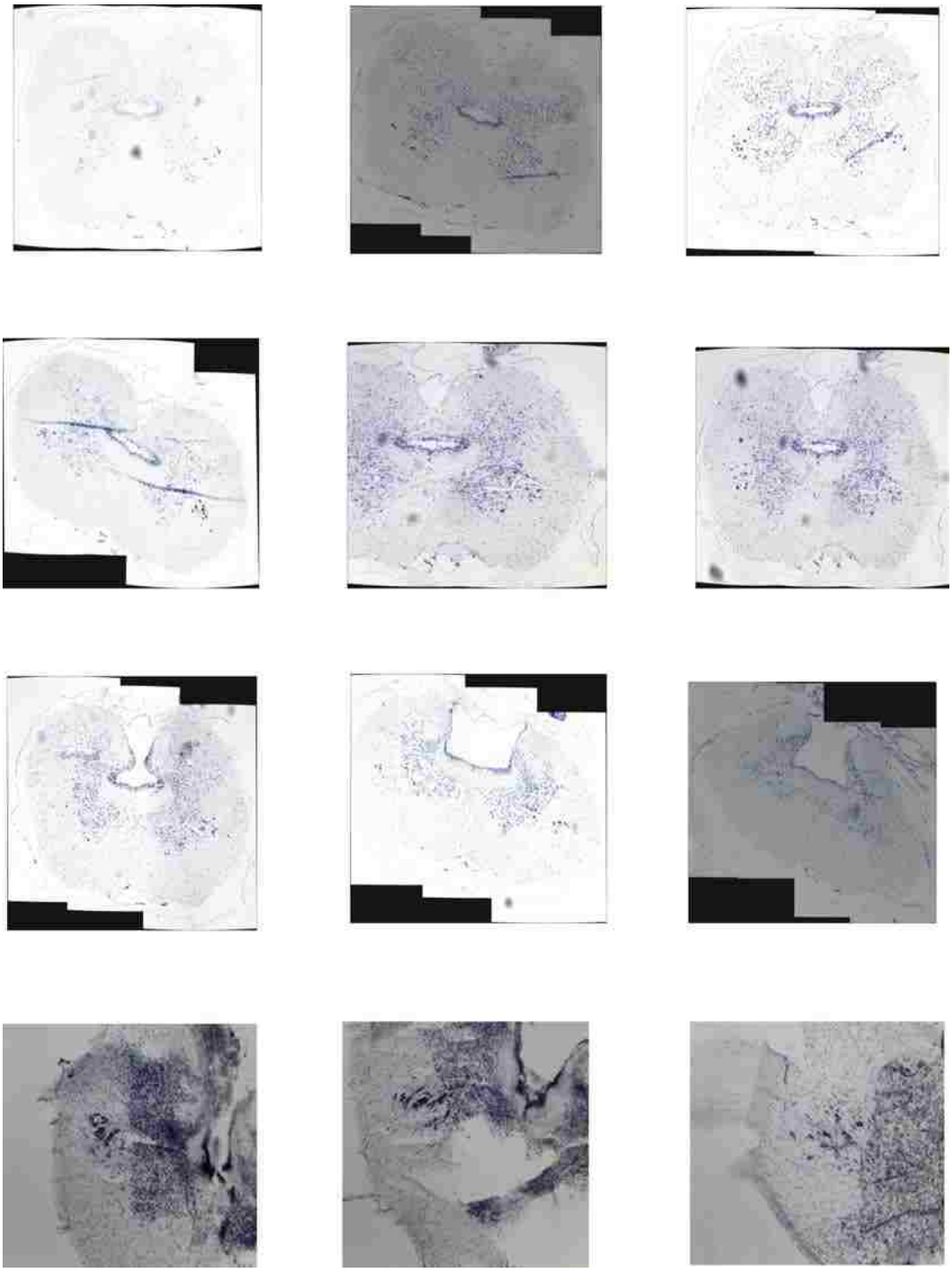








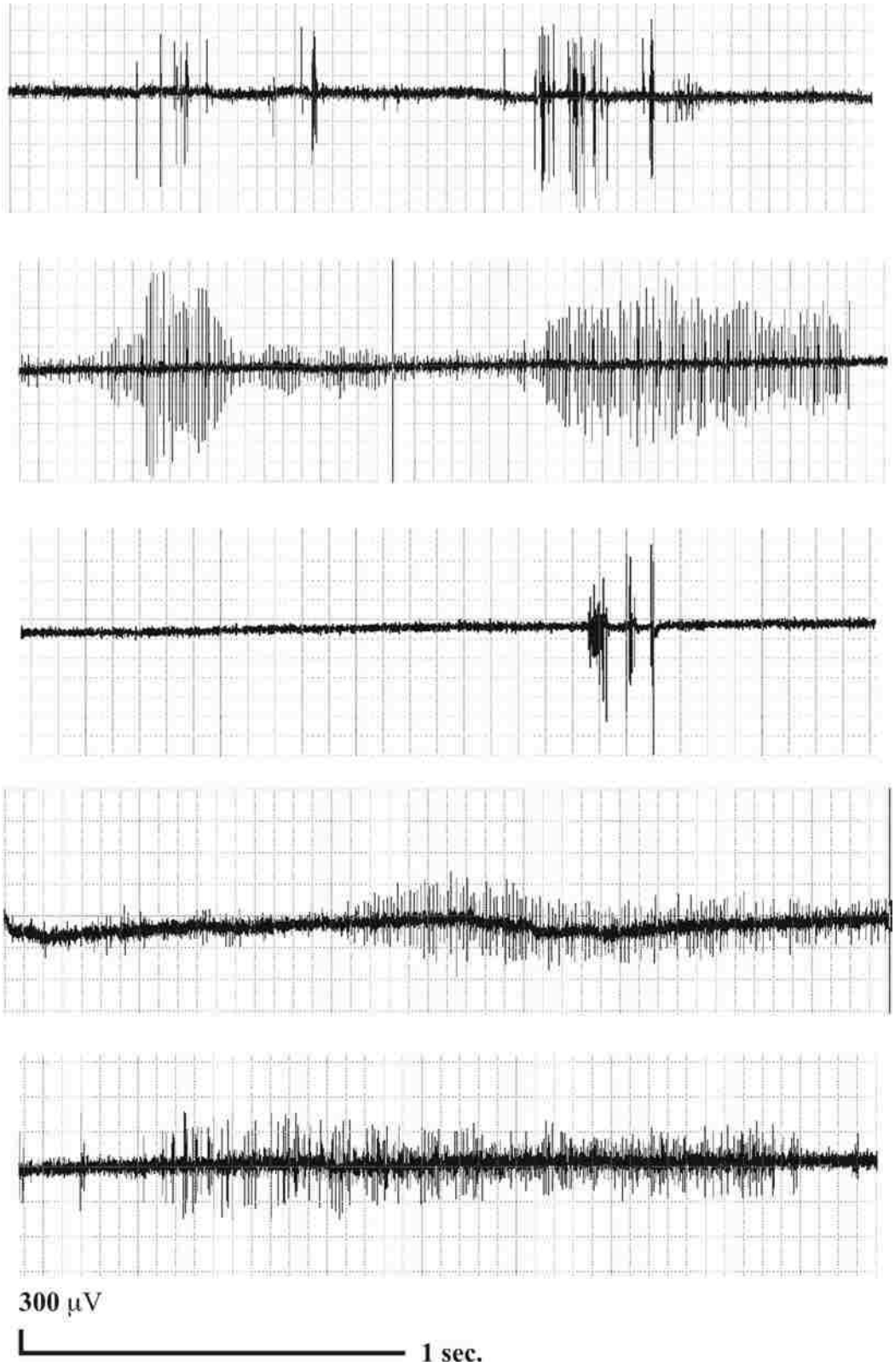
Cresyl Violet Stain:



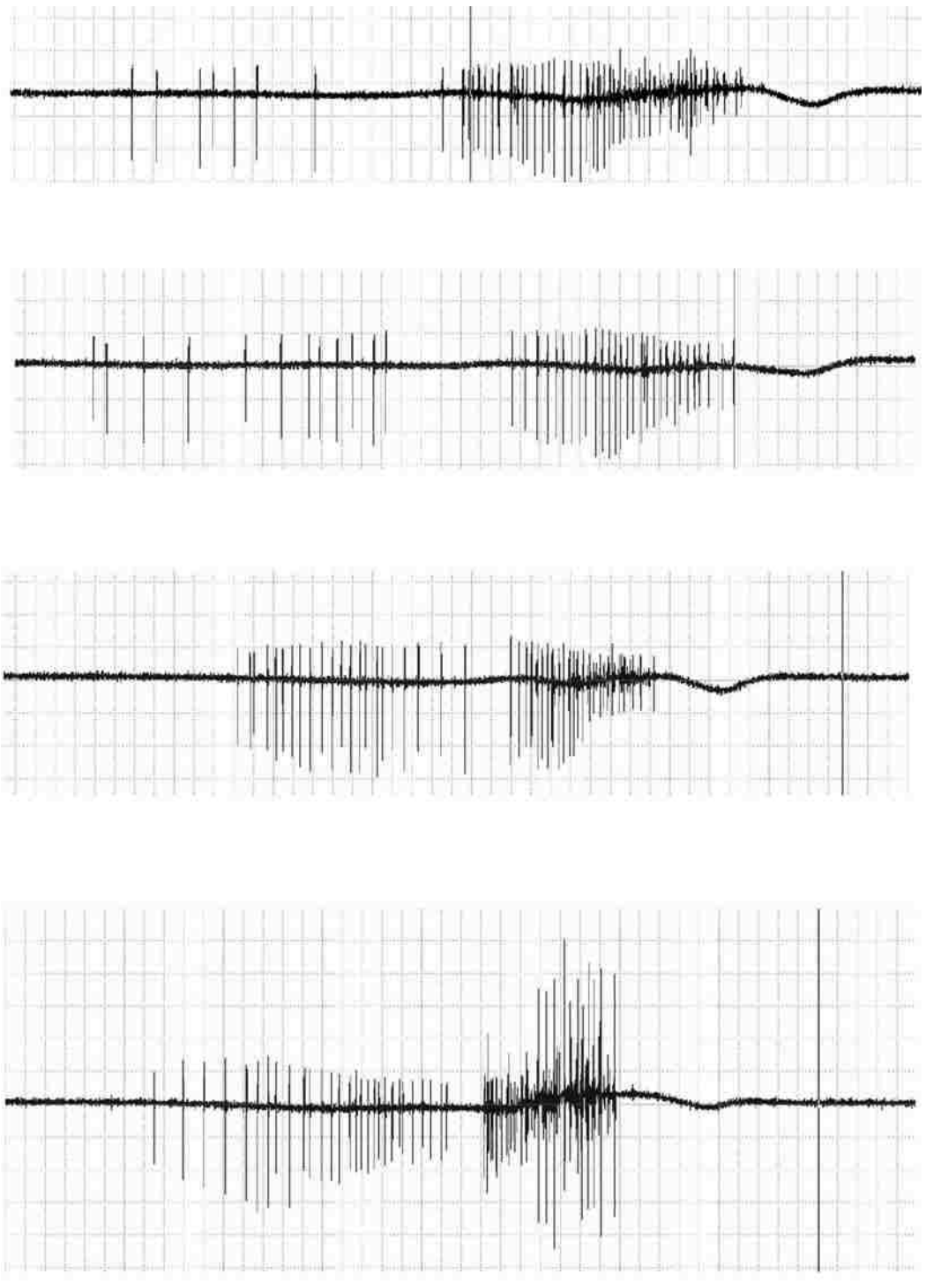
APPENDIX IV
ELECTROMYOGRAM (EMG) RECORDINGS IN PBDE-209 TREATED AFRICAN CLAWED FROGS

X. laevis vocalizations were recorded using EMG. A pair of wires was implanted deep within the laryngeal dilator muscle so that each muscle contraction and click vocalization could be recorded. Representative EMGs show differences in vocalization among PBDE-209 treated male frogs. As PBDE-209 concentration was increased, vocal ability decreased in frequency of calls and click amplitude. For each recording, scalebars represent 300 microvolts for amplitude and 1 second for call duration.

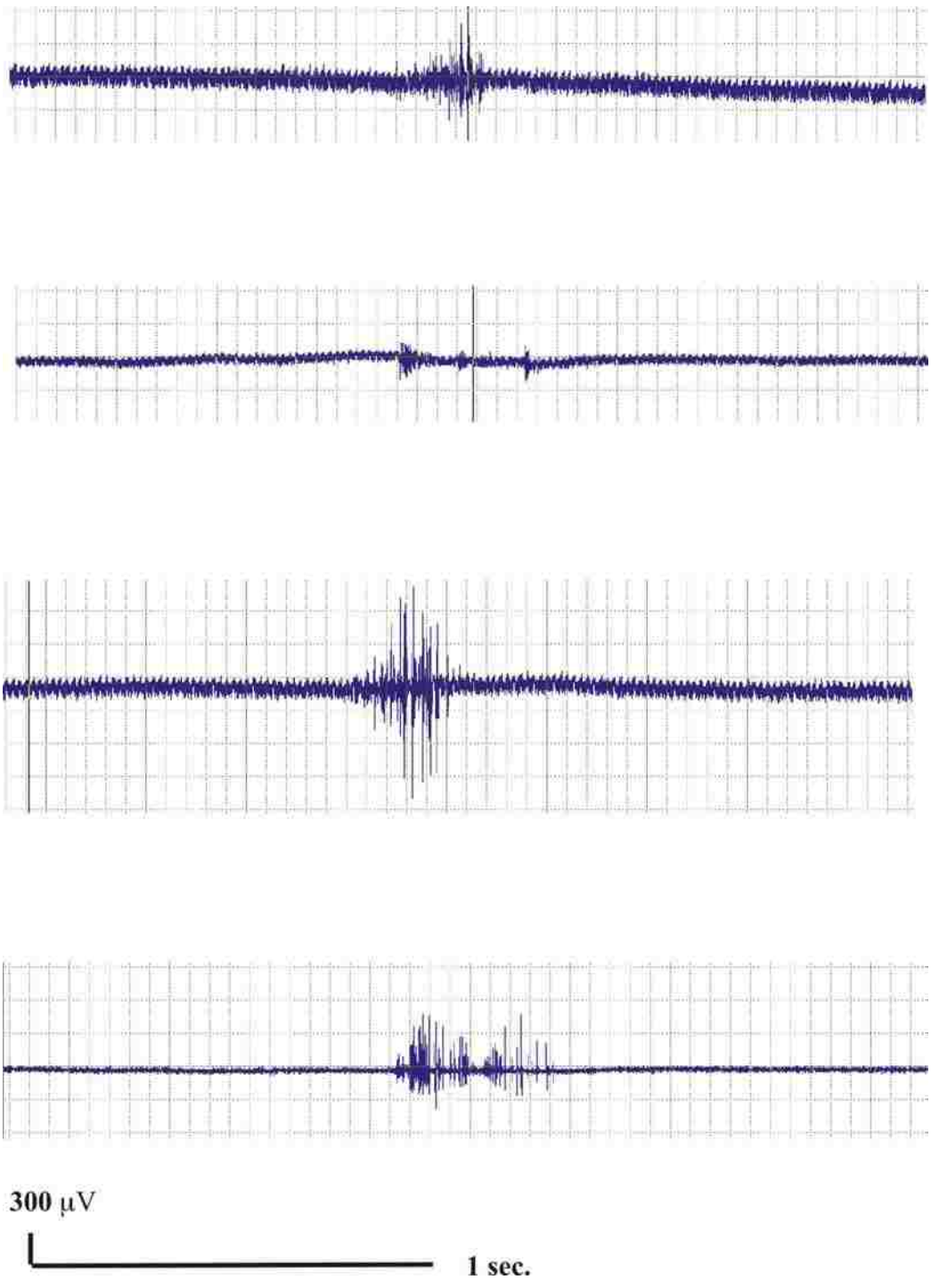
Control Calls:



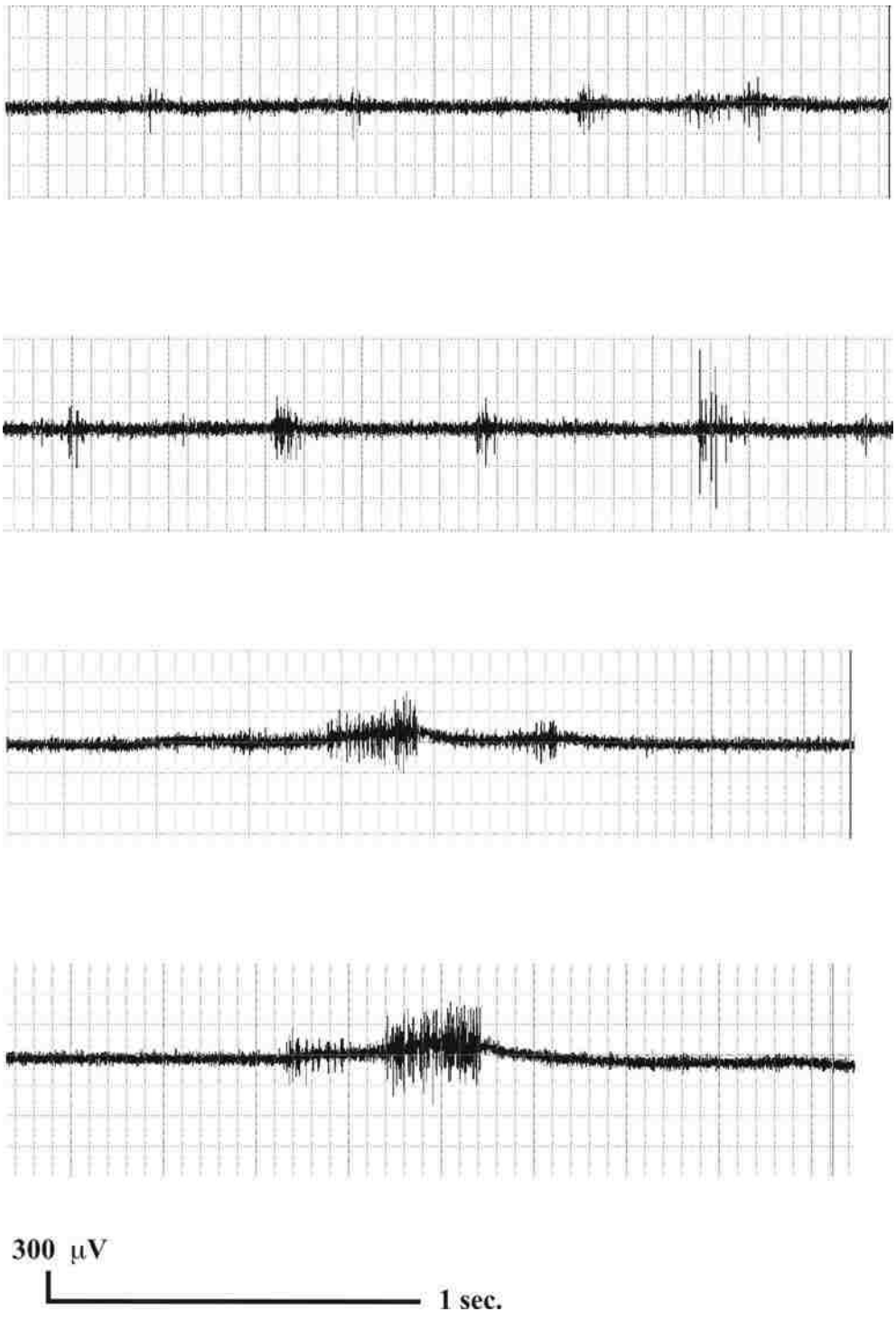
Control Calls:



Low Dose Calls:



Middle Dose Calls:



High Dose Calls:

VITA

Lisa Rania Ganser was born in January 1972 to Francisco and Brigida Rania of Muskegon, Michigan. She graduated in 1994 with Honors in Biology research from Saint Mary's College, Notre Dame, Indiana. Following her undergraduate career, Lisa spent a year doing research with the University of Georgia's Savannah River Ecology Lab in Aiken, South Carolina then as a laboratory instructor at her undergraduate alma mater. In 1998 Lisa graduated with a Master of Science degree from Dr. Catherine Propper's comparative endocrinology laboratory at Northern Arizona University in Flagstaff, Arizona. After a year-long stint as a histologist at the Mayo Clinic in Rochester, Minnesota and a four-year temporary position as a Biology, Anatomy, and Physiology instructor at Saint Mary's College, Lisa began her Ph.D. program at University of Miami in Dr. Jim O'Reilly's laboratory. Lisa moved to the Auditory Neurobiology lab or Dr. John Lu in 2004 and received her Ph.D. in Biology in May 2009.

Permanent Address: 18121 SW 137th Ct. Miami, FL 33177