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A handwritten signature in cursive script, reading "Tara Romanczyk". The signature is fluid and elegant, with a long, sweeping underline that extends to the right.

ABSTRACT

Our understanding of the cellular and molecular mechanisms underlying adult neurogenesis in response to diseases of the CNS is incomplete. Decreased neurogenesis in the adult hippocampus in response to stressors is well established in rodent and nonhuman primate models of depression. Recently, increased neurogenesis has been linked to the actions of certain antidepressants, including tranylcypromine (TCP) in the treatment of depression. The goldfish (*Carassius auratus*) has the ability to grow its body and brain throughout its life and is an ideal model for studying adult neurogenesis. The hypothesis was that alterations in adult goldfish proliferation zones can be used to determine antidepressant effects on proliferation. Three specific aims were used to test this hypothesis: 1) To produce an atlas of the goldfish brain based on cresyl violet stained sections. 2) To construct an atlas of proliferation zones in the goldfish brain. 3) To study the influence of the antidepressant tranylcypromine on progenitor cell proliferation zones in the telencephalon, optic tectum, and cerebellum of adult goldfish.

Proliferation zones were identified using BrdU and H3 immunohistochemistry and mapped using the brain atlas. Proliferation zones were associated with periventricular surfaces in the telencephalon, diencephalon, optic tectal lobes, cerebellum, and hindbrain. The presence of proliferation zones within the hindbrain was one region that differed from other teleosts.

The effects of TCP on proliferating cells in the adult goldfish brain were examined. TCP caused a significant increase in cell proliferation in the optic tectum and cerebellum as detected by BrdU immunohistochemistry. Comparison of the treatment paradigms determined that short term TCP treatment caused significantly more proliferation than long term TCP treatment when examined at 16 hour BrdU administration. The significant decrease in BrdU labeled cells observed in the long term TCP treatment may be attributed to apoptosis, a process coupled with neurogenesis. These results demonstrate that the goldfish is as an excellent vertebrate model for investigating the mechanisms involved in antidepressant effects on the brain.

**EVALUATION OF THE ADULT GOLDFISH BRAIN AS A MODEL FOR THE
STUDY OF PROGENITOR CELLS**

by

Tara B. Romanczyk

Dissertation submitted to the faculty of the

Program in Neuroscience of the

Uniformed Services University of the Health Sciences

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Dedication

I would like to dedicate this thesis to my parents Jan and Lee Romanczyk. Thank you so much for everything! Words can hardly describe my thanks and appreciation to you. You have been my source of inspiration, support, and guidance. You have taught me to be unique, determined, to believe in myself, and to always persevere. I am truly thankful and honored to have you as my parents. To take a quote from Albert Schweitzer, “At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us.” You, mom and dad, have been that spark for me when my light blew out. Thank you for your unwavering love and support along this journey I have taken. I love you both always and forever.

The Road Not Taken

Robert Frost, Mountain Interval. (1920).

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I —
I took the one less traveled by,
And that has made all the difference.

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LIST OF ABBREVIATIONS

AC	Anterior Commissure
ANOVA	Analysis of Variance
AP	Area Postrema
ASC	Adult Stem Cell
ASPA	Aspartoacylase
ATN	Anterior Tuberal Nucleus (Nucleus Tuberalis Anterior)
BC	Brachial Conjunctivum
BDNF	Brain Derived Neurotrophic Factors
BSA	Bovine Serum Albumin
BrdU	Bromodeoxyuridine
CC	Cerebellar Crest (Crista Cerebelli)
CCEG	Granular Layer of Cerebellar Corpus (Corpus Cerebelli, Stratum Granulosum)
CCML	Molecular Layer of Cerebellar Corpus (Corpus Cerebelli, Stratum Moleculare)
c-FLIP	Cellular FLICE-Inhibitory Protein
cm	Centimeter
CNP	2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase
CNS	Central Nervous System
CP	Central Posterior Thalamic Nucleus (Nucleus Centralis Posterior Thalami)

CY3	Cyanine 3
d	Day
DAB	3,3' Diaminobenzidine
DAPI	4, 6- Diamino-2-Phenylidole
Dc	Central zone of dorsal telencephalic area (area dorsalis telencephali, zona centralis)
DCX	Doublecortin
Dd	Dorsal Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Dorsalis)
DF	Nucleus Diffusus of the Inferior Lobe
DH	Dorsal Horn
dH ₂ O	Distilled Water
DiV	Diencephalic Ventricle
Dm	Medial Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Medialis)
DMNX	Dorsal Motor Nucleus of the Vagus
DNA	Deoxyribonucleic Acid
DP	Posterior Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Posterior)
DTN	Dorsal Tegmental Nucleus (Nucleus Tegmentalis Dorsalis)
Dv	Ventral Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Ventralis)
E	Entopeduncular Nucleus (Nucleus Entopeduncularis)

ESC	Embryonic Stem Cell
FITC	Fluorescein Isothiocyanate
FR	Habenulointerpeduncular Tract (Fasciculus Retroflexus = Tractus Habenulointerpeduncularis)
FZ	Fiber Zone
g	Gram
GE	Granular Eminence (Ementia Granularis)
GFAP	Glial Fibrillary Acidic Protein
GN	Glomerulosis Nucleus
5HT	Serotonin
H	Habenular Nucleus (Nucleus Habenularis)
HCl	Hydrochloric Acid
HC	Horizontal Commissure
Hc	Caudal Zone Of Periventricular Hypothalamus (Nucleus Periventricularis Hypothalami, Zona Caudallis)
Hd	Dorsal Zone Of Periventricular Hypothalamus (Nucleus Periventricularis Hypothalami, Zona Dorsalis)
HNO ₃	Nitric Acid
HuC/D	Anti HuC/HuD Neuronal Protein
[H ³]-thymidine	Tritiated Thymidine
Hv	Ventral Zone of Periventricular Hypothalamus (Nucleus Periventricularis Hypothalami, Zona Ventralis)
IACUC	Institutional Animal Care and Use Committee

IC	Internal Cellular Layer of Olfactory Bulb (Stratum Cellulare Internum Bubli Olfactorii)
Ig	Immunoglobulin-like
IMRF	Intermediate Reticular Formation (Formatio Reticularis, Pars Intermedia)
i.p.	Intra-peritoneal
LCAG	Granular Layer of Caudal Lobe of Cerebellum (Valvula Cerebelli, Pars Medialis, Stratum Granulosum)
LFB	Lateral Forebrain Bundle (Fasciculus Lateralis Telencephali)
LH	Lateral Hypothalamic Nucleus
LL	Lateral Longitudinal Fascicle = Lateral Lemniscus (Fasciculus Longitudinalis Lateralis)
LMVC	Molecular Layer of the Lateral Part of the Valvula Cerebelli (Lobus Caudalis Cerebelli, Stratum Moleculare)
LRN	Lateral Recess Nucleus (Nucleus of the Lateral Recess)
LOT	Lateral Olfactory Tract (Tractus Olfactorius Lateralis)
LPG	Lateral Preglomerular Nucleus (Nucleus Praeglomerular Lateralis)
LPS	Lipopolysaccharide
LSD1	Lysine Specific Demethylase 1
LVII	Facial Lobe (Lobus Facialis)
LX	Vagal Lobe (Lobus Vagus)
MAP-2	Microtubule Associated Protein 2

MB	Mammillary Body (Corpus Mamillare)
MFB	Medial Forebrain Bundle (Fasciculus Medialis Telencephali)
mL	Milliliter
MLF	Medial Longitudinal Fascicle (Fasciculus Longitudinalis Medialis)
μm	Micrometer
mm	Millimeter
MOA	Monoamine Oxidase
MON	Medial Octavolateralis Nucleus (Nucleus Octavolateralis Medialis)
MOT	Medial Olfactory Tract
MPN	Medial Pretoral Nucleus
MPP ⁺	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MZ	Motor Zone
NaOH	Sodium Hydroxide
NDM	Dorsomedial Nucleus of the Thalamus
NE	Norepinephrine
NeuN	Neuronal Nuclei
NF-70	Neurofilament-70
NLT	Lateral Tuberal Nucleus (Nucleus Lateralis Tuberis)
NLV	Lateral Valvula Nucleus

NMLF	Nucleus of the Medial Longitudinal Fascicle (Nucleus Fasciculi Longitudinalis Medialis)
nm	Nanometer
NR	Nucleus Rotundus
NS	Normal Serum
OA	Olfactory Afferent Fibers
OC	Optic Chiasm (Chiasma Opticum)
OCT4	Octamer- Binding Transcription Factor - 4
OLIG-2	Oligodendrocyte Transcription Factor 2
ON	Optic Nerve (Nervus Opticus)
OT, OTe	Optic Tectum (Tecti Optici)
PB	Phosphate-Buffered
PBS	Phosphate- Buffered Saline
PC	Posterior Commissure
PCD	Programmed Cell Death
PG	Preglomerular Complex
PGZ	Periventricular Gray Zone of Optic Tectum (Stratum Periventriculare Tecti Optici)
PPa	Parvocellular Preoptic Nucleus, Anterior Part (Nucleus Praeopticus Parvocellularis, Pars Anterior)
PPd	Periventricular Pretectal Nucleus , Dorsal Part (Nucleus Praetectalis Periventricularis, Pars Dorsalis)
PPN	Posterior Periventricular Nucleus

RT	Room Temperature
RV	Rhombencephalic Ventricle (Ventricularis Rhombencephali)
SEM	Standard Error of the Mean
SOX2	SRY-Related HMG Box 2
SVZ	Subventricular Zone
SZ	Sensory Zone
T	Tangential Nucleus (Nucleus Tangentialis)
TCP	Tranylcypromine
TeV	Tectal Ventricle (Ventricularis Mesencephali)
TGN	Tertiary Gustatory Nucleus
TL	Longitudinal Torus
TNF- α	Tumor Necrosis Factor-Alpha
TP	Posterior Tuberal Nucleus
TPp	Periventricular Nucleus of Posterior Tuberculum (Nucleus Periventricularis Tuberculi Posterioris)
TuJ1	β -III Tubulin
TUNEL	Terminal dUTP nick-end labeling
V	Ventricle
Vcm	Medial Lobe of Valvula of Cerebellum
Vd	Dorsal Zone Of Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Dorsalis)
VDi	Intermediate Subnucleus of Vd
VH	Ventral Horn

VII	Sensory Root of the Facial Nerve
VIII	Octaval Nerve (Nervus Octavus)
VI	Lateral Nucleus Of The Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Lateralis)
VLG	Granular Layer of the Lateral Part of the Valvula Cerebelli (Valvula Cerebelli, Pars Medialis, Stratum Granulosum)
Vm	Ventromedial Thalamic Nucleus (Nucleus Ventromedialis Thalami)
VMG	Granular Layer of the Lateral Part of the Valvula Cerebelli (Valvula Cerebelli, Pars Medialis, Stratum Granulosum)
VMM	Molecular Layer of the Medial Part of the Valvula Cerebelli (Valvula Cerebelli, Pars Medialis, Stratum Moleculare))
Vp	Postcommissural Nucleus of Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Postcommissuralis)
Vs	Sensory Root of the Trigeminal Nerve (Radix Motoria Nervi Trigemini)
Vv	Ventral Zone of Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Ventralis)
WHO	World Health Organization
X	Vagal Nerve (Nervus Vagus)
Xm	Motor Root of the Vagal Nerve
Xs	Sensory Root of the Vagal Nerve

CHAPTER 1

INTRODUCTION

A Brief History of Neurogenesis

A central dogma of neurogenesis for nearly a century was that no new neurons were formed after birth [1]. Scientists had agreed that in adult mammals, if nerve cells in the brain died, they were not replaced [1]. Based on this concept, it was believed that adult mammals were born with a maximum number of neurons and that this number continuously decreased throughout the life of the animal [1]. However, the existing neurons did have the capacity to make new synapses [1]. Suggestions regarding the existence of dividing cells in the postnatal CNS were raised in the early nineteen hundreds [2]. However, it was impossible, using methods of the time, to trace the fate of those rare dividing cells and to test the hypothesis that the newborn cells were in fact neurons rather than glia [3].

In the late 1950s, a new method was developed that used tritiated thymidine ($[H^3]$ -thymidine) to label dividing cells [4]. Tritiated thymidine was incorporated into replicating DNA during the S-phase of the cell cycle and could be detected by autoradiography [4]. Soon after, a series of papers were published by Altman and colleagues reporting $[H^3]$ -thymidine evidence for new neurons in various brain regions including the dentate gyrus of the hippocampus, neocortex, and olfactory bulb [5-7]. Unfortunately, these reports were largely ignored since they were considered to lack functional relevance. Adult neurogenesis was revisited

in the late 1970s when Kaplan and Hinds [8] demonstrated that newborn neurons in the rodent hippocampus survived for long periods of time, extended axons and appeared to receive synaptic inputs [9].

The field was revolutionized in 1982 when Gratzner introduced the first monoclonal antibody to 5- Bromo-2'-deoxy-uridine (BrdU) [10, 11]. It was not until the last decade of the twentieth century that adult neurogenesis finally gained acceptance due to the identification and isolation of adult neural stem cells from the central nervous system (CNS) of rodents [12-14].

Adult Neurogenesis

Historically, stem cell biology was restricted to embryology and to the biology of organs with inherent regenerative ability such as the hematopoietic system [15]. Only with the demonstration of precursor cells in organs originally considered to be non-regenerating, such as the lung, liver, and finally, the heart and brain, were stem cells viewed as a ubiquitous phenomenon in the body [16-27].

“Adult neurogenesis” is the production of new neurons in the adult brain. The term “neurogenesis” literally means “birth of new neurons” [28]. However, adult neurogenesis is much more complicated and encompassing, as it is a process and not an event. Neurogenesis is not only the proliferation of new cells, but also encompasses migration, differentiation and survival of the progeny.

A brain region that can generate neurons is called a “neurogenic” region [1, 29]. Neurogenic implies two processes: first, the presence of immature

precursor cells from which new neurons can develop, and second, a microenvironment that is permissive for neurogenesis to occur [29]. In the adult mammalian brain, there are two known neurogenic regions, the hippocampus and the olfactory system [1]. The rest of the brain is categorized as “non-neurogenic” [30]. Neurogenic regions have been found in mammals, birds, amphibians, reptiles and fish [1]. In the teleost, a group of fish that belong to the infraclass Teleostei, which includes the goldfish, there are many regions throughout the entire brain categorized as neurogenic. These include the telencephalon (containing olfactory bulbs and frontal lobes), optic tectum, cerebellum and vagal lobes [31]. Non-neurogenic regions of the CNS, under normal conditions do not display neurogenesis but might do so under special conditions, such as exposure to growth factors or following an injury such as stroke [30, 32].

Cardinal Features of Stem and Progenitor Cells

There is a broad array of terminology and definitions surrounding stem cells. A stem cell is defined as a cell that has the ability to divide or self-replicate to make exact copies or clones of itself for an indefinite period and can differentiate into other cell types that generate the organism [33]. Stem cells possess two properties that make them particularly useful for neural replacement: self-renewal and terminal differentiation of the stem cell or its progeny [16]. Differentiation involves the progressive restriction of a cell’s developmental or stem cell properties, while increasing its specialization of function [16].

There are two main types of stem cells: embryonic and adult stem cells.

An embryonic stem cell (ESC) is an undifferentiated, pluripotent cell derived from the inner cell mass of the blastocyst, which is part of the early (day 4-5) embryo [34]. ESCs are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm, and they are the ancestors to primordial germ cells and all tissue-specific fetal and adult stem cells.

The adult stem cell (ASC), sometimes referred to as somatic stem cell, is an undifferentiated, unspecialized cell that occurs in differentiated tissue and is able to self-renew, but, unlike ESCs, ASCs give rise to only the specialized cell types from which they originated under in vivo conditions [33]. In a living organism, the primary roles of ASCs are to maintain and repair the tissue in which they are found, however, their origins remain elusive. To date, ASCs have been derived from brain [16], bone marrow [17], blood [18], dental pulp [19], skeletal muscle [20], skin [21], adipose tissue [22], retina [23], liver [24, 25], and pancreas [26] [33] [27].

ASCs in the mammalian brain, including mice, rats, guinea pigs, rabbits, primates, and humans, are present in the hippocampal dentate gyrus and the subventricular zone (SVZ) [1]. The SVZ is part of the rostral migratory stream to the olfactory system, where ASCs constitute a small population (0.1-1%) of relatively quiescent cells [35, 36]. ASCs isolated from the SVZ in the wall of the lateral ventricle of the brain divide in response to epidermal growth factor and basic fibroblast growth factor [36]. The cells of the SVZ generate both neuronal and glial progeny. However, it remains debatable whether neural stem cells

reside in the SVZ itself, or in the overlying ventricular lining called the ependyma [36]. The proliferative capacity of ependymal cells remains controversial [37, McKay, 1999 #497]. The ependyma has all the features of a differentiated, post-mitotic epithelium. However, the cells present in this layer also express immature neural markers, consistent with a stem cell function [36]. Johansson *et al.* proposed that the ependymal stem cell divides asymmetrically to form one daughter cell, which stays undifferentiated, and another cell that moves into the subventricular layer below [36, 38]. Further investigation into the mechanistic actions and generation of neural ASCs is needed.

A progenitor cell is defined as a cell that is lineage restricted, partially specialized and maintains the ability to divide or self-replicate to produce two daughter stem cells, two daughter progenitor cells or one of each [1, 39]. Progenitor cells possess limited self-renewal capability and are multipotent [16, 40-42]. A multipotent cell can give rise to several lineage restricted cell types, but those types are limited in number [16, 40-42].

Goldfish, Neurogenesis

Neurogenesis in the mammalian CNS is typically considered to be a limited developmental process. Adult neurogenesis becomes more region-specific as development progresses [43].

Post-embryonic mammalian CNS neurogenesis is a relatively rare occurrence, primarily restricted to the olfactory system, hippocampus, and more recently found in the visual cortex and mammalian forebrain [16, 44-47]. Smaller

vertebrates, such as songbirds, have a greater capacity for post-natal neurogenesis [48]. Additionally, the amphibian brain has the capability to regenerate and regain function following injury [49]. The teleost infraclass is one of the few vertebrate taxa that are capable of regenerating whole brain structures after partial removal of brain regions [50]. Goldfish (*Carassius auratus*), belong to the teleost family and can regenerate neural tissues in the adult [51, 52]. Consequently, the goldfish offers a unique model for understanding morphogenetic events such as proliferation, growth control, and differentiation.

Teleosts, which include the goldfish and other fish mentioned throughout this thesis, belong to the infraclass Teleostei, in class Actinopterygii [53]. This diverse group arose during the Triassic period and approximately 20,000 species in about 40 orders are still in existence [53]. Teleosts have modifications in jaw musculature, including a movable maxilla and premaxilla, which make it possible for teleosts to protrude their jaws outwards from the mouth [54]. Additionally, the caudal fin's upper and lower lobes are about equal in size, and the spine ends at the caudal peduncle, the narrow part of the fish's body to which the tail fin is attached [54].

Goldfish and other teleosts maintain stem cell populations throughout their lives and can regenerate neural tissues in the adult brain [51, 52]. In post-embryonic goldfish, cell proliferation (including, but not limited to, neuronal proliferation) occurs at the crescent-shaped rim of the rostral optic tectum, a region known as the germinal zone [52, 55]. Although older goldfish have larger numbers of neurons in the region, the relative rate of neuron addition is lower in

these fish as compared to younger ones, suggesting that the rate of neurogenesis slows down [52, 56, 57].

In another teleost, the brown ghost knifefish (*Apteronotus leptorhynchus*), 25% of new brain cells generated in the adult are located in the telencephalon, diencephalon, mesencephalon, and rhombencephalon, while 75% are located in the cerebellum [58, 59]. Newly generated cells in the cerebellum migrate to the associated granule cell layers (rostral cerebellum) or to the eminentia granularis pars posterior (caudal cerebellum). Once at their target region, approximately 50% of the newly generated cerebellar cells undergo apoptosis [60]. The remaining cells survive for the rest of the fish's life, thus contributing to permanent brain growth [60].

Neuronal replacement in the goldfish is dependent on recruitment of stem cells from the proliferative zones (also referred to as germinal zones or matrix zones) [55]. These stem or progenitor cells can be induced to differentiate into neurons and glia [13, 16, 38, 61, 62]. The progeny are located in proliferative zones adjacent to the ventricles, paraventricular and cisternal systems, or in areas that are derived from proliferation zones located at ventricular surfaces during embryonic development [58-60, 63-67]. The majority of cells born in such proliferation zones migrate to specific target areas within a few days following their generation [65, 67]. These newly generated cells have the capability to survive for the greater part of the life span of the fish, if they do not undergo apoptosis immediately following migration [68, 69]. The concept that proliferative zones may exist as remnants of embryonic proliferative zones is not new, nor is it

species specific. It was suggested by Opalski (1933) [70] and Kershman (1938) [71] that in mammals, the subependymal layer (subventricular zone (SVZ)) persists into adulthood as a smaller vestigial layer and retains the ability to produce new cells [72].

The advantages of using the goldfish model for the study of neurogenesis rather than the mammal is that the goldfish brain maintains stem cell populations throughout their lives and can regenerate neural tissues in the adult brain, while neurogenesis in the mammal is more restricted [51, 52]. The goldfish has more regions of proliferation [31] than does the mammal, which is limited to just two regions, the dentate gyrus of the hippocampus and the SVZ of the lateral ventricle, which is part of the rostral migratory stream [36]. Additionally, they are inexpensive, readily available, and easy to maintain. The zebrafish, although similar to the goldfish, has primarily been used for developmental studies and genetic screening [1]. Adult neurogenesis was first reported in the goldfish in 1977 [73], while surprisingly, it wasn't until 2001 that adult neurogenesis was first reported in the zebrafish [74]. The goldfish has been used since 1867 as a test model for determining the toxicity of various substances [75]. Therefore, the goldfish would serve to as a reliable model for drug testing including antidepressants.

Research specifically on goldfish neurogenesis is limited and centered predominantly on retinal and optic nerve regeneration [52, 56]. The first well-studied example of neurogenesis and repair in the goldfish involved the retina. Sperry and his colleagues [76] found that a ring of stem cells surrounded the

retina, and the progeny of these stem cells migrated into a damaged region and reestablished connections with the optic tectum of the goldfish brain.

CNS regeneration post-injury occurs in other teleost species. Three concentrated proliferation zones, the dorsal matrix zone, caudal matrix zone, and basal matrix zone, are instrumental for the regeneration of the optic tectum in carp (*Carassius carassius*) [31]. Neuronal and glial cells are continually developed from undifferentiated matrix (stem/progenitor) cells in these regions, producing an increase in the tectal size of the fish throughout its lifespan, in addition to aiding post-injury regeneration [52, 57, 77]. Cellular differentiation rates in these zones increase following tectal lesioning. An older paper by Seegar [31] described ablation work by two researchers (Kirsche and Marón) on the regenerative ability of the carp brain. Kirsche [78], examined regeneration of specific regions in the optic tectum following ablation and found the presence of “matrix tissue” to be necessary for regeneration. Marón [79], investigated regeneration in another teleost, guppy or rainbowfish (*Lebistes reticulatus*) and found that after the 120th day following ablation, the telencephalon appeared to be almost normally developed. Studies in other teleosts reveal that post-injury regeneration is not limited to the optic tectum. For instance, the brown ghost knifefish had displayed gradual neuronal regeneration and recovery following stab wound to the corpus cerebelli (CCb) [58, 69]. In another study of the brown ghost fish, cerebella were stabbed and the role of cell proliferation in neuronal regeneration was investigated. Proliferative activity was up-regulated approximately 2.5 times in the injured cerebellar region as compared to its

normal control counterpart, and could be seen up to 28 days post-lesioning [58, 69]. Neurogenesis is known to occur in the adult goldfish brain; however an atlas of proliferative zones does not exist. An atlas of the forebrain does exist for the goldfish [120]; however an atlas of the remainder of the goldfish brain does not. The ablation of focal and global brain regions in the goldfish has indicated the presence of proliferative zones along with the potential for CNS regeneration and repair [31, 52, 57, 77].

Depression, Antidepressants and Neurogenesis

Depression is a devastating illness that affects an estimated 21 million American adults, or approximately 10% of the population [80]. Approximately half of those will have major or clinical depression. According to the Centers for Disease Control and Prevention's (CDC) National Center for Health Statistics, intentional self-harm (suicide) was the 11th leading cause of death in 2004 [81]. Women are twice as likely to suffer from depression as men. Unfortunately, about two-thirds of people suffering from depression do not seek treatment [82]. By the year 2020, the World Health Organization (WHO) estimates that depressive disorder will become the number two most prevalent cause of illness-produced disability [83].

Antidepressants are typically prescribed for the treatment of depression and other affective disorders. However, the mechanism(s) of how these agents exert their therapeutic effects are poorly understood. Generally, antidepressants work to increase levels of monoamines, in particular, dopamine (DA),

norepinephrine (NE) and serotonin (5-HT). The increase in monoamines caused by antidepressants suggests that a biochemical imbalance within these monoaminergic systems may underlie the pathogenesis of depression and provides the rationale for the monoaminergic hypothesis of depression. This hypothesis proposes that depression is due to a deficiency in monoaminergic neurotransmission [84, 85].

Depression is thought to have distinct morphological correlates in the brain and it has recently been linked to adult neurogenesis in the hippocampus [83]. A new scenario being considered is that the hippocampus is part of a functional neural network that involves the amygdala, anterior cingulate, orbitofrontal and dorsolateral cortex, striatum and the medial temporal lobe [83]. Over the past ten years, there has been a developing hypothesis linking depression and declining neurogenesis. Depression and declining neurogenesis may be causally connected, which would imply that depressive symptoms could arise from impairments in neurogenesis. Antidepressant action could be involved in the alleviation of symptoms of depression.

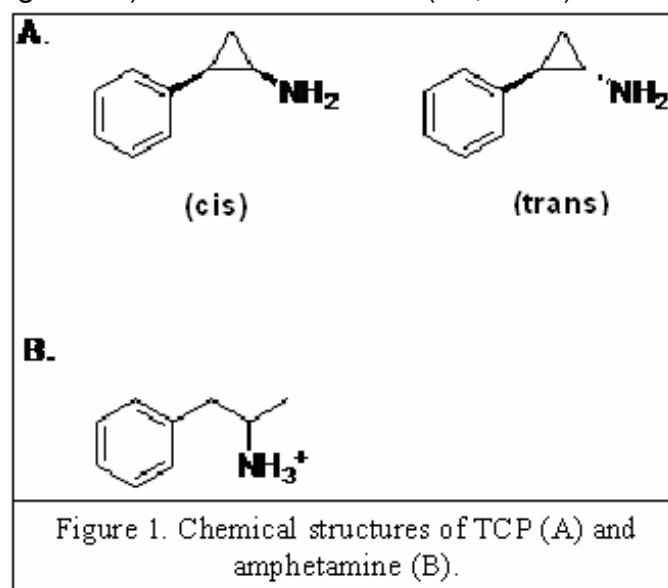
Depression symptoms include but are not limited to a disturbance of mood, movement (either excessive or slowed physical movement) and cognition [86]. These symptoms are predominantly associated with structures in the proposed neural network described above. One definitive cause of depression that is known to affect the hippocampus in mammals is stress. Stress and depression have been intertwined throughout evolutionary biology [87]. Stress is capable of precipitating depressive episodes in humans and can lead to cell death, dendritic

shrinkage, and decreased levels of neurotrophins, such as brain derived neurotrophic factor (BDNF) within the hippocampus [85]. Chronic antidepressant treatment has been shown to block the stress-induced downregulation of BDNF expression in the hippocampus [88]. Recently, stress was shown to decrease adult neurogenesis in the hippocampus and chronic antidepressant treatment was shown to increase it, suggesting these processes to be involved in the pathogenesis and treatment of depression [85, 89-91].

Tranylcypromine

All major classes of antidepressants, including the specific drugs TCP [89, 90], fluoxetine [89, 90] and imipramine [89, 90], have recently been shown to increase neurogenesis in adult rat hippocampi [89, 90] and the non-human primate [91]. The antidepressant tranylcypromine (TCP), a nonselective irreversible inhibitor of monoamine oxidase (MAO), an enzyme found in the outer mitochondrial membrane that breaks down the neurotransmitters dopamine, norepinephrine, and serotonin, has been used to treat depression in humans. TCP has been utilized as an effective antidepressant drug for over forty years [92]. TCP has recently been used in the treatment of Parkinson's disease, a disease that is associated with the loss of DA producing neurons within the substantia nigra pars compacta, in both humans [92-94] and goldfish [95].

Interestingly, tranylcypromine (Figure 1A) is a stereoisomeric (cis, trans) substance, or racemic mixture, structurally analogous to amphetamine (Figure 1B). TCP is structurally analogous to amphetamine, as TCP was synthesized in an attempt to develop a less problematic stimulant. Amphetamine is a



highly addictive psychostimulant with weak MAO inhibitory properties while TCP is a potent and clinically useful MAO inhibitor but is only mildly stimulating and weakly addictive [96]. Based on the optical activity of TCP, it has been found that (+) – TCP mainly inhibits MAO while (-) – TCP interacts with monoamine-reuptake and release [97]. Although TCP is a stereoisomer the individual isomers have not been investigated separately in the treatment of depression or Parkinson's disease. Additionally, TCP treatment leads to an increase in trace amines which are believed to partially play a role in the pathophysiology of depression [97].

TCP has been shown to increase the number of BrdU positive cells in the mammalian hippocampus [89]. In addition to TCP inhibiting MAO, TCPs mechanism of action in neurogenesis has recently been established in mammalian embryonal carcinoma cells, another type of stem cell derived from a type of cancer called teratocarcinoma. Briefly, TCP inhibits the flavo- enzyme

lysine specific demethylase 1 (LSD1). The function of LSD1 is to demethylate lysine 4 on Histone 3. By blocking this enzyme, TCP leaves Histone 3 methylated, thereby activating the pluripotential stem cell marker *OCT-4* and other stem cell-specific transcription factors [98]. However, the effect of TCP on telostean neurogenesis has not been examined.

Role of Apoptosis in the Brain

One question that arises with the production of new cells in the postnatal brain is their functional and anatomical destiny. Based on many reports investigating neurogenesis, the majority of newly produced cells in the teleost undergo apoptosis [58, 68, 99-103]. This apoptosis is necessary for regulation of neural growth.

Cell death is an active, genetically programmed process, which is regulated by pro- and anti-apoptotic genes, and disruptions to this process can lead to disease [104, 105, 106]. Kerr *et al.* first described the active process of cell death known as apoptosis in 1972 [107]. Professor James Cormack proposed the term “apoptosis” (ἀπόπτωση), which is the Greek word to describe the “dropping off” or “falling off” of petals from flowers, or leaves from trees [107].

Apoptosis, also referred to as programmed cell death (PCD), is a highly regulated process in the normal development of both vertebrates and invertebrates. That regulation balances cell proliferation and cell death in order to maintain tissue or organ size and function. For example, skin, intestinal epithelial cells, olfactory epithelium, liver, blood and immune system cells, are

cells that have a finite life, die by PCD, and are replaced by new cells following stem cell division and differentiation [108].

Historically, interest in apoptosis as a key developmental phenomenon evolved as the adaptive role(s) by PCD in morphogenesis and histogenesis became a major research interest [109-111]. The biological role of developmental apoptosis (e.g. effect of cell death on proliferating or newly differentiated cells), compared to the molecular and disease-related aspects of PCD, had largely been ignored until recent years [112].

PCD has been more extensively reported on in mammals and birds, while little is known in reptiles, amphibians and the teleost fish. Therefore, much of what is known is based on a handful of animal models. PCD occurs in neurons and glia of the CNS in the major subtypes of cells (motor neurons [113], sensory neurons [114, 115], sensory receptors [116], and interneurons [117], as well as Schwann cells [118] astrocytes [119], and oligodendrocytes [120]. For neurons in the CNS, the timing of apoptosis occurs prior to the onset of connectivity, and involves progenitor or undifferentiated cells, as well as during synaptogenesis, when neurons are differentiating [108].

Although adult neurogenesis may be beneficial, it is restricted to specific brain regions. In mammals, adult neurogenesis is mainly constrained to two brain regions, the dentate gyrus (DG) of the hippocampus and the olfactory system [121-123]. While adult neurogenesis produces new neuronal cells, it does so at the expense of the already established and functional circuitry. Therefore, in order to circumvent this potential pitfall, it is necessary for adult

neurogenesis to be accompanied by apoptosis. To address this problem, PCD could be used as a strategy to eliminate newly born or mature cells that fail to functionally integrate into existing circuitry. Additionally or alternatively, PCD may function to modulate neuronal migration, presumably by creating space for new cells or by chemoattractants secreted by dying neurons or other cell types (e.g., phagocytic microglia) [108].

While neurogenesis is tightly restricted to two predominant regions in the adult mammalian brain, it is widespread in the goldfish brain. This family of fish has long-lasting brain growth and a high capacity for regeneration [65]. Due to this resource of progenitor cells, the goldfish is as an excellent model to use to investigate the mechanisms governing antidepressant effects on neurogenesis and the fate of the progeny. Following is the hypothesis for this study and the specific aims used to test this hypothesis.

Hypothesis: Alterations in the proliferation zones of the adult goldfish brain can be used to determine antidepressant effects on proliferation.

Specific Aims:

- 1) To produce an atlas of the goldfish brain based on cresyl violet stained sections.
- 2) To construct an atlas of proliferation zones in the goldfish brain.
- 3) To study the influence of the antidepressant tranylcypromine on progenitor cell proliferation zones in the telencephalon, optic tectum, and cerebellum of adult goldfish.

CHAPTER 2

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

A total of 100 adult goldfish (*Carassius auratus*) were used in this study under an approved USUHS Institutional Animal Care and Use Committee (IACUC) protocol. Goldfish of both sexes were obtained from a local aquarium dealer and maintained in aerated and filtered water. Fish were approximately 7 cm in length and weighed 4.5 ± 1.5 g. All fish were maintained in the tanks for a minimum of two weeks prior to the experiments and were fed fish chow to satiation once daily. The fish were maintained on a 12- hour light/ dark photoperiod.

Euthanasia Methods

Individual fish used to create the goldfish brain atlas were euthanized by transcardial perfusion of aldehydes. Fish were anesthetized with a 0.02% solution of 3-Aminobenzoic Acid Ethyl Ester (a.k.a. MS222 or methanesulfonate salt) (Sigma, St. Louis, MO) in 1 L of distilled water (dH₂O) [124] until tail pinch response was absent. Each fish was placed ventral side up on top of gauze wetted with anesthetic solution, and a lavage was inserted into the mouth with a constant anesthetic drip. A longitudinal incision was made from the ventral (pelvic) fins to just below the pectoral fins to expose the heart. The goldfish heart, unlike the mammalian heart, consists of three chambers: bulbus

arteriosus, atrium, and ventricle. The perfusion fixation method developed by Laties et. al. [125] was used with minor modifications for the goldfish. Briefly, the atrium was cut to open the circulatory system, and a 25-gauge needle was inserted into the ventricle. The tissue was then fixed by transcardial perfusion with 4% paraformaldehyde (room temperature (RT)). Four percent paraformaldehyde was prepared by adding 12 g of paraformaldehyde (Sigma) to 150 ml of dH₂O and heated to 60-68 °C while stirring. Once at temperature, the solution was removed and 1 M sodium hydroxide (NaOH) (Sigma) was added drop-wise until the solution cleared. An equal volume of 0.2 M phosphate buffer (PB) (0.78 g of sodium phosphate monobasic (Sigma) and 6.48 g of sodium phosphate dibasic (Sigma) in 150 mL of dH₂O) was combined with the paraformaldehyde solution and stirred. The pH was adjusted to 7.2 with NaOH if too acidic or 1 N hydrochloric acid (HCl) (Sigma) if too basic.

For immunohistochemical procedures, fish were euthanized by decapitation. Goldfish were placed onto a piece of gauze. Decapitation was performed by making a cut from the dorsal aspect of the body with a pair of scissors, aligned behind the gill plate. The head was quickly cut off.

Fixation and Post-Fixation Techniques

A total of 20 goldfish were used for this part of the study. Several goldfish brain fixation and post-fixation techniques were investigated in order to establish optimal results for cresyl violet staining and BrdU immunohistochemistry. The fixation methods included: (a) transcardial perfusion with 4% paraformaldehyde

followed by post fixation in 4% paraformaldehyde (30 minutes, 4 or 24 hours, 4 °C); (b) transcardial perfusion with 10% formalin (Sigma) followed by post-fixation in 10% formalin (30 minutes, 4 or 24 hours, 4 °C); and (c) rapid freeze (-87 °C) in an acetone-dry ice slurry. Following fixation methods (a) and (b), brains were cryoprotected in 20% sucrose in PB (4 °C) until the brains sank, indicating that the sucrose had replaced the water. Twenty percent sucrose was made by adding 20 g of sucrose (Sigma) to 100 mL of 0.2 M phosphate buffer (0.52 g of sodium phosphate monobasic (Sigma) and 4.32 g of sodium phosphate dibasic (Sigma) in 100ml of dH₂O). The solution was mixed until the sucrose was dissolved. Other post-fixation methods were tried, including: paraffin embedding, dehydration plus paraffin embedding, and submersion of the unfixed brain in 20% sucrose (4 °C).

Following the various fixation and post-fixation methods, the brains were subsequently cryostat (-18 °C) sectioned and processed for cresyl violet staining or BrdU immunohistochemistry. For all methods, the tissue was assessed for morphological quality and positive label. Based on these two criteria, two techniques were selected for implementation. Transcardial perfusion with 4% paraformaldehyde followed by a post-fixation in 4% paraformaldehyde (30 minutes, 4 °C), and cryoprotection in 20% sucrose (4 °C) were used to fix the goldfish brains. This fixation procedure was used for the construction of the goldfish brain atlas, which consisted of cresyl violet stained sections. The rapid freeze method in which brain tissue was placed in an acetone-dry ice slurry was used to prepare the brains for all immunohistochemical examinations.

Sectioning

After euthanasia and tissue processing, goldfish brains were sectioned on a Hacker-Bright OTF cryostat (Hacker Instruments Inc., Fairfield, NJ) at 20 μm for the preparation of the goldfish brain atlas and mapping of the progenitor zones. Alternatively, brains were sectioned at 30 μm for single and double label immunohistochemical and tranylcypromine protocols. Sections were cut and mounted serially on gelatin coated slides (Thomas® red label® micro-slides, Thomas Scientific, Swedesboro, NJ). Gelatin coated slides were prepared by washing uncoated slides that were placed into a metal slide rack in 9% Nitric Acid (HNO_3) (Sigma) by dipping up and down (5 times). The slides were then rinsed in dH_2O that was changed several times to ensure complete HNO_3 removal, and then placed onto absorbent paper. The coating solution was prepared by heating 1 L dH_2O to boiling, to which gelatin (3 g, Sigma) was added first, followed by chromium potassium sulfate (0.5 g, Sigma) while stirring. Slides were dipped into the subbing solution (4 times), excess solution drained off, followed by placement onto absorbent paper to dry (1 hour) and repeated once. Lightly covered slides were dried (overnight, room temperature (RT)). The following day, slide racks were tightly wrapped in plastic wrap.

The preparation of serially mounted sections consisted of mounting one section per slide onto a group of ten slides in numerical order (Figure 2). This procedure resulted in each slide having one section. This process was repeated for the next thirty serial sections resulting in each slide having four sections. This

process was repeated on groups of 10 slides until all of the serial sections from an entire goldfish brain (frontal lobes through to the vagal lobes) were mounted.

Slide# 1	Slide# 2	Slide# 3	Slide# 4	Slide# 5	Slide# 6	Slide# 7	Slide# 8	Slide# 9	Slide# 10
21 31 1 11	22 32 2 12	23 33 3 13	24 34 4 14	25 35 5 15	26 36 6 16	27 37 7 17	28 38 8 18	29 39 9 19	30 40 10 20

Slide# 11	Slide# 12	Slide# 13	Slide# 14	Slide# 15	Slide# 16	Slide# 17	Slide# 18	Slide# 19	Slide# 20
61 71 41 51	62 72 42 52	63 73 43 53	64 74 44 54	65 75 45 55	66 76 46 56	67 77 47 57	68 78 48 58	69 79 49 59	60 70 40 50

Figure 2. Schematic of slide labeling.

GOLDFISH BRAIN ATLAS

A total of five adult goldfish were used to create an atlas of the goldfish brain based on cresyl violet stained sections. Following staining, every fifth section was digitally captured at 2 X with a Sony (Tokyo, Japan) DKC 5000 Catseye digital still camera (1,544 x 1,120 x 3 pixels) connected to a Nikon (Tokyo, Japan) biophot microscope. These images were then imported into Adobe® Photoshop® 5.5 (Adobe Systems Incorporated, San Jose, CA), saved in TIFF format and labeled. The TIFF images were imported into Microsoft® Office PowerPoint® (Microsoft Corporation), where slides were created, labeled and printed. Because no complete goldfish brain atlas existed, brain regions were identified by studying other available brain sections obtained from published papers on various teleost species including, but not limited to: the goldfish [52, 126-132], carp [133-135], zebrafish [67, 102, 136-139], three-spined stickleback [63], knifefish [58-60, 65, 101, 140], and others [141-143].

Perfusions

To create an atlas of the goldfish brain, fish were anesthetized with a 0.02% solution 3-Aminobenzoic Acid Ethyl Ester (a.k.a. MS222 or methonesulfonate salt) (Sigma) in 1 L of dH₂O until an absence of the tail pinch was observed, followed by euthanization by transcardial perfusion with 4% aldehyde (as described under Euthanasia methods, page 16).

After fixation, the brain was immediately removed and placed into 4% paraformaldehyde (30 minutes, 4 °C). The brain was cryoprotected in 20% sucrose in PB. The brains remained in sucrose (24 - 48 hours) until they sank indicating that they were saturated with sucrose.

Sectioning

After euthanasia and processing of the goldfish brain, tissue was sectioned on a Hacker-Bright OTF cryostat (Hacker Instruments Inc., Fairfield, NJ) at 20 µm. Serial sections were mounted on gelatin coated slides as described under Sectioning, page 19.

Cresyl Violet Staining

Cresyl violet stains the basophilic components of neurons, neuroglia, ependymal, and endothelial cells of blood vessels. Within neurons, it stains the nucleus, nucleolus, and Nissl substance. Tissue sections were stained with cresyl violet (Sigma) [144] for three seconds followed by a rinse in water. The

sections were air-dried, placed briefly into xylene and coverslipped with Permount (Sigma).

MAPPING OF PROLIFERATION ZONES

Bromodeoxyuridine Fluorescent Immunohistochemistry

To generate maps of proliferation zones in the goldfish brain, BrdU (Sigma) was used to label mitotic cells. The cells were plotted throughout the brain (frontal lobes through cerebellum) onto adjacent cresyl violet stained sections.

A total of three goldfish were used to perform fluorescent immunohistochemistry to map the proliferation zones. Goldfish were placed into 1 L of tank water containing 5 mM BrdU (Sigma), and allowed to swim freely for 24 hours [145]. Over the 24 hour labeling period, the cells which labeled mitotic cells that had undergone one or two cell cycles of division [146]. After 24 hours, goldfish brains were excised, placed onto a 1 cm x 2 cm copper wire mesh and rapidly frozen in an acetone-dry ice slurry (-87 °C). Brain tissue was sectioned at 20 µm on a Hacker-Bright OTF cryostat (Hacker Instruments Inc., Fairfield, NJ) and serially mounted on gelatin coated slides (as described under Sectioning on page 19).

DNA was denatured by incubating the sections in 0.1 N HCl (5 minutes, 37 °C) [147]. The acid was neutralized with 0.1 M borate buffer (pH 8.5, 5 minutes, RT) and washed in 0.01 M phosphate buffered saline (PBS) (Sigma) (3 x 3 minutes). Sections were then treated with primary antibody monoclonal rat-anti

BrdU (1:100; Accurate Chemical & Scientific Corp, Westbury, NY) (overnight, 4 °C). Sections were washed with PBS (3 x 3 minutes, RT), and then treated with Fluorescein (FITC)-conjugated AffiniPure goat- anti –rat (1:200), or Cy3-conjugated AffiniPure goat- anti -rat (1:1000) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) secondary antibody (30 minutes, RT). The sections were washed in PBS (1 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium (Vector Laboratories, Burlingame, CA).

Identification of Progenitor Zones by Mapping of BrdU Positive Cells

To analyze BrdU fluorescent immunohistochemical sections, every fifth section was examined throughout the brain of three goldfish using a Nikon (Tokyo, Japan) Labophot fluorescent microscope. BrdU positive cells were plotted throughout the brain directly onto printed images of the respective adjacent cresyl violet stained sections (frontal lobes through cerebellum, using the brain atlas as a guide for neuroanatomy).

The plotted images from all three goldfish were then merged together using transparencies that were placed on top of the corresponding section in the brain atlas. The resulting plots were then scanned (EPSON Perfection 3170 photo scanner). These images were imported into Adobe® Photoshop® 5.5 (Adobe Systems Incorporated) and saved in TIFF format. The TIFF images were imported into Microsoft® Office PowerPoint® (Microsoft Corporation), where slides were created, labeled and printed.

IMMUNOHISTOCHEMICAL PROCEDURES

A total of 12 goldfish were euthanized by decapitation (as described under euthanasia page 16). The brains were quickly excised and rapidly frozen in an acetone-dry ice slurry. The frozen brains were then placed into Tissue-Tek® OCT Compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and stored at -20 °C until processed for cryostat sectioning. After euthanasia and tissue processing, brain tissue was sectioned on a Hacker-Bright OTF cryostat (Hacker Instruments Inc.).

Controls for Immunohistochemistry

Control slides were performed concurrently with various antibody(ies) examined for all immunohistochemistry. Positive controls were performed using mouse brain tissue. Ferret cortical tissue was used as a positive control for the BrdU peroxidase immunohistochemistry. Negative controls were performed by omitting the primary antibody(ies).

Single Label Fluorescent Immunohistochemistry

Single label immunohistochemistry was performed with various antibodies. These antibodies were used to identify mitotic cells, stem cells, progenitor cells, postmitotic neurons and glia (including astrocytes and oligodendrocytes) within the goldfish brain. Tables containing specific details of antibodies used in the sections to follow are listed in appropriate sections (see pages 26, 32, and 36)

Images of single label fluorescent immunohistochemistry were digitally captured (10 X, 20 X, 40 X, 60 X, 63 X, and/ or 100 X) with a Sony DKC 5000 Catseye digital still camera connected to a Nikon Labophot fluorescent microscope. Adobe® Photoshop® 5.5 was used to label and print images.

Mitotic, Stem and Progenitor Markers

Table1. Antibodies Used To Identify Mitotic, Stem and Progenitor Cells.

Mitotic/ Stem Cell Markers	Concentration/ Description	Company	Labels
Bromodeoxy-uridine	1:100 Rat monoclonal	Accurate Chemical & Scientific Corp.	Detects BrdU incorporation into DNA in place of thymidine; Used for immunocytochemical analysis of cell proliferation. Nuclear label.
Anti- Phosphohistone H3 (Ser10)	1:100 Mouse monoclonal	Cell Signaling Technology	Detects histone H3 only when phosphorylated at serine10. Nuclear label.
Anti-Proliferating Cell Nuclear Antigen	1:500 Mouse monoclonal	Sigma	PCNA is a marker for cells in early G1 phase and S phase of the cell cycle. Nuclear label.
	1:100 Goat polyclonal	Santa Cruz	
Anti- Pax-6, C-Terminus	1:500 Mouse monoclonal	Chemicon	PAX6 is a transcription factor with important functions in the development of the central nervous system (CNS). (stem cell marker) Nuclear label.
Anti- Pax-6, Clone AD1.5	1:500 Mouse monoclonal	Chemicon	
Ki67	1:50 Rabbit polyclonal	Abcam	The prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase). Routinely used as a neuronal marker of cell cycling and proliferation. Nuclear label.
Anti-Nanog	1:500 Rabbit polyclonal	Abcam	Homeodomain-bearing transcriptional factor. Expression is specific to early embryos and pluripotential stem cells. Key molecule involved in the signaling pathway for maintaining the capacity for self-renewal and pluripotency. Nuclear label.
Anti- Octamer -4	1:500 Rabbit polyclonal	Abcam	Transcription factor expressed by undifferentiated embryonic stem and germ cells. Nuclear label.
Anti- SRY Related HMG BOX Gene 2	1:500 Rabbit polyclonal	Abcam	Transcription factor (ESC marker) Nuclear label.
Progenitor Marker	Concentration/ Description	Company	Labels
Anti-Nestin	1:100 Mouse monoclonal	Chemicon	Nestin is a Class VI intermediate filament widely used as a predominant marker for stem / progenitor cells, in the mammalian CNS. Cytoplasmic label.

Anti-Phosphohistone H3 (Ser10)

Brain sections (30) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% normal serum (NS) (Jackson ImmunoResearch Laboratories, Inc)/ 0.3% Triton X-100 (Sigma, 1 hour, RT), and incubated with primary antibody (Phospho-Histone H3 (H3) (1:100, Cell Signaling Technology, Danvers, MA)), in 0.01 M PBS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS (3 x 5 minutes, RT), then incubated in 0.01 M PBS/ 0.2% Triton X-100, with Alexa Fluor 488 goat- anti- mouse IgG (1:100; Invitrogen, Carlsbad, CA) secondary antibody (1 hour, RT). Slides were washed in 0.01 M PBS (3 x 5 minutes) and coverslipped using VECTASHIELD® Mounting Medium with 4', 6-diamino-2-phenylidole (DAPI) medium, which is a nuclear stain that emits blue fluorescence upon binding to adenosine-thymine (AT) regions of DNA (Vector Laboratories) [148].

Anti-Proliferating Cell Nuclear Antigen

Two protocols were examined for this procedure. Procedure 1: Brain sections (25) were post-fixed in cold (-20 °C) acetone (5 minutes, RT), then air dried (30 minutes, RT). Slides were rinsed in 0.01 M PBS/ 0.1% bovine serum albumin (BSA)/ 0.1% Triton X-100, blocked in 0.01 M PBS/ 10% NS/ 0.1% BSA/ 0.1% Triton X-100 (10 minutes, 4 °C), and incubated in primary antibody (monoclonal proliferating cell nuclear antigen (PCNA) (1:500, Sigma, or 1:100, Santa Cruz Biotechnology Inc., Santa Cruz CA), in 0.01 M PBS/ 0.1% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 0.1% BSA/ 0.1%

Triton X-100, then incubated in 0.01 M PBS/ 0.1% BSA/ 0.1% Triton X-100 (30 minutes, RT), with FITC-conjugated AffiniPure goat- anti- mouse IgG (Jackson ImmunoResearch Laboratories, Inc.), Alexa Fluor 594 goat- anti- mouse IgG (1:100; Invitrogen), or Cy3- conjugated AffiniPure donkey- anti- goat IgG (1:1000; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody. Slides were washed in 0.01 M PBS (3 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Procedure 2: Brain sections (10) were washed in 0.01 M PBS (2 x 5 minutes, RT). Antigen retrieval was performed by placing slides into 0.1M sodium citrate buffer (29.41 g citric acid (tri sodium salt, dihydrate), in 1 L dH₂O, pH 6.0) heated (15 minutes, 85 °C). Slides were cooled to RT, then washed in 0.01 M PBS (2 x 2 minutes, RT) blocked in 0.01 M PBS/ 10% NS/ 0.1% BSA/ 0.1% Triton X-100 (10 minutes, 4 °C), and incubated in primary antibody (proliferating cell nuclear antigen (PCNA) (1:3000 Sigma, (1:100), Santa Cruz Biotechnology Inc.), in 0.01 M PBS/ 0.1% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 0.1% BSA/ 0.1% Triton X-100 (4 x 5 minutes, RT), then incubated in 0.01 M PBS/ 0.1% BSA/ 0.1% Triton X-100 (30 minutes, RT), with FITC-conjugated AffiniPure goat- anti- mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.) or Cy3- conjugated AffiniPure donkey- anti- goat IgG (1:1000; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody. Slides were washed in 0.01 M PBS (3 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti- Pax-6

Brain sections (15) were rinsed in 0.01 M PBS, and fixed in 4% paraformaldehyde (10 minutes, RT). Antigen retrieval was performed by placing slides into heated 0.1M sodium citrate buffer (10 minutes, 70 °C). Slides were then incubated in the primary antibody (Mouse anti- Pax-6, C-terminus or anti- Pax-6, clone AD1.5 monoclonal antibody (1:500 Chemicon)) in 0.01 M PBS/ 5% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 0.2% Triton X-100 (2 x 5 minutes, RT) and incubated in 0.01 M PBS/ 0.2% Triton X-100 (30 minutes, RT) with Alexa Fluor 488 goat- anti-mouse (1:100; Invitrogen) secondary antibody. Slides were washed in 0.01 M PBS/ 0.2% Triton X-100, (2 x 5 minutes, RT), then 0.01 M PBS (2 x 5 minutes, RT) and coverslipped using VECTASHIELD[®] Mounting Medium with DAPI (Vector Laboratories).

Anti- Ki67

Brain sections (15) were washed in 0.01 M PBS/ 0.1% BSA/ 0.1% Triton X-100 (4 x 5 minutes, RT), blocked in 0.01 M PBS/ 10% NS/ 0.1% BSA/ 0.1% Triton X-100 (10 minutes, 4 °C) and incubated in the primary antibody (Ki67 (1:50), Abcam, Cambridge, MA)), in 0.01 M PBS/ 0.1% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 1% BSA/ 0.1% Triton X-100 (4 x 5 minutes, RT), and incubated in 0.01 M PBS/ 0.1% Triton X-100, with FITC-conjugated AffiniPure goat- anti- rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody (30 minutes, RT). Slides were washed in

0.01 M PBS (3 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-Nanog

Brain sections (15) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% normal serum (NS) / 0.2% Triton X-100 (1 hour, RT), and incubated in primary antibody (Nanog (1:100, Abcam)) in 0.01 M PBS/ 3% NS/ 0.3% Triton X-100 (overnight, 4° C). Slides were washed in 0.01 M PBS/0.2% Triton X-100 (2 x 5 minutes, RT), blocked in 0.01 M PBS/ 5% NS/ 0.2% Triton X-100 (30 minutes, RT), and then incubated in 0.01 M PBS/ 0.2% Triton X-100 (30 minutes, RT), with Alexa Fluor 488 goat- anti- rabbit IgG (1:100; Invitrogen) secondary antibody. Slides were washed in 0.01 M PBS/ 0.2% Triton X-100 (4 x 5 minutes, RT), followed by a wash in 0.01 M PBS (4 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-Octamer-4

Brain sections (15) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% normal serum (NS) / 0.2% Triton X-100 (1 hour, RT), and incubated in primary antibody (Octamer-4 (Oct4) (1:500, Abcam) in 0.01 M PBS/ 3% NS/ 0.3% Triton X-100 (overnight, 4° C). Slides were washed in 0.01 M PBS/0.2% Triton X-100 (2 x 5 minutes, RT), blocked in 0.01 M PBS/ 5% NS/ 0.2% Triton X-100 (30 minutes, RT), and then incubated in 0.01 M PBS/ 0.2% Triton X-100 (30 minutes, RT), with Alexa Fluor 488 goat- anti- rabbit IgG (1:100; Invitrogen, Carlsbad, CA)

secondary antibody. Slides were washed in 0.01 M PBS/ 0.2% Triton X-100 (4 x 5 minutes, RT), followed by a wash in 0.01 M PBS (4 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti- SRY related HMG BOX gene 2

Brain sections (15) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% normal serum (NS) / 0.2% Triton X-100 (1 hour, RT), and incubated in primary antibody SRY related HMG BOX gene 2 (Sox2) (1:500, Abcam) in 0.01 M PBS/ 3% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/0.2% Triton X-100 (2 x 5 minutes, RT), blocked in 0.01 M PBS/ 5% NS/ 0.2% Triton X-100 (30 minutes, RT), and then incubated in 0.01 M PBS/ 0.2% Triton X-100 (30 minutes, RT), with Alexa Fluor 488 goat- anti- rabbit IgG (1:100; Invitrogen) secondary antibody. Slides were washed in 0.01 M PBS/ 0.2% Triton X-100 (4 x 5 minutes, RT), followed by a wash in 0.01 M PBS (4 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Progenitor Marker

Anti-Nestin

Brain sections (15) were washed in 0.01 M PBS/ 0.1% BSA/ 0.1% Triton X-100 (4 x 5 minutes, RT), blocked in 0.01 M PBS/ 10% NS/ 0.1% BSA/ 0.1% Triton X-100 (10 minutes, 4 °C) and incubated in the primary antibody (Nestin

(1:100), Chemicon), in 0.01 M PBS/ 0.1% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 1% BSA/ 0.1% Triton X-100 (4 x 5 minutes, RT), and incubated in 0.01 M PBS/ 0.1% Triton X-100, with Alexa Fluor 488 goat- anti-mouse IgG (1:100; Invitrogen) secondary antibody (30 minutes, RT). Slides were washed in 0.01 M PBS (3 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Neuronal markers

Table2. Antibodies Used To Identify Neuronal Cell.

Neuronal Markers	Concentration/ Description	Company	Labels
Anti HuC/ HuD Neuronal Protein (HuC/D)	1:100 Mouse monoclonal	Invitrogen	RNA-binding protein that recognizes neuronal proteins. Labeling seen in neurons that leave the mitotic cycle (post-mitotic immature neurons) Cytoplasmic label.
Anti-Doublecortin	1:3000 Guinea pig polyclonal	Chemicon	Doublecortin is expressed in migrating and differentiating neuronal cells.
	1:3000 Goat polyclonal	Santa Cruz	Cytoplasmic label.
Anti-Microtubule- Associated Protein 2	1:500 Mouse monoclonal	Sigma	MAP2 is the major microtubule associated protein of brain tissue. Expressed in mature neurons. Cytoplasmic label.
Anti-Neuronal Nuclei	1:300 Mouse monoclonal	Chemicon	Recognizes the DNA-binding, neuron-specific protein NeuN, present in most CNS postmitotic neuronal cell types. Staining is primarily localized in the nucleus of the neurons with lighter staining in the cytoplasm.
Anti-Neurofilament- 70	1:100 Mouse monoclonal	Chemicon	Intermediate filament proteins found in axons of large projection neurons. Cytoplasmic label.
Anti-βIII Tubulin	1:1000 Mouse monoclonal	Covance	Expressed during fetal and postnatal development. Transient expression is also present in the subventricular zones of the CNS comprising putative neuronal- and/or glial precursor cells.
	1:200 Mouse monoclonal	Promega	In adult tissues, the distribution of beta III is almost exclusively neuron-specific.
	1:100 Mouse monoclonal	Sigma	Cytoplasmic label.

Anti HuC/ HuD Neuronal Protein (HuC/ D)

Brain sections (30) were rehydrated in 0.01 M PBS (40 minutes, RT).

Antigen retrieval was performed by submerging the slides into 50 mM Tris HCl (0.61 g Tris base (Sigma) in 100 mL dH₂O, pH adjusted with concentrated HCl to pH 8.0) heated to 70 °C (30 minutes). Slides were cooled to RT, washed in 0.01 M PBS/ 0.1% Triton X-100 (1 x 10 minutes, RT), washed in 0.01 M PBS (2 x 5 minutes, RT), then blocked in 0.01 M PBS/ 10% NS/ 1% BSA/ 0.1% Triton X-100 (30 minutes, 4 °C). Slides were incubated with the primary antibody (anti HuC/ HuD neuronal protein (HuC/D) (1:100, Invitrogen)) in 0.01 M PBS/ 1% BSA (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 1% BSA (3 x 10 minutes, RT), and incubated in 0.01 M PBS/ 1% BSA/ 0.1% Triton X-100 (20 minutes, RT), with Alexa Fluor 488 goat- anti- mouse IgG (1:100; Invitrogen) secondary antibody. Slides were washed in 0.01 M PBS (2 x 10 minutes, RT) and coverslipped using VECTASHIELD[®] Mounting Medium with DAPI (Vector Laboratories).

Anti-Doublecortin

Brain sections (15) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% NS/ 0.3% Triton X-100 (30 minutes, RT), and incubated with the primary antibody (guinea pig anti-doublecortin (DCX) polyclonal (1:3000, Chemicon); or goat anti-DCX polyclonal (1:3000, Santa Cruz Biotechnology Inc.)) in 0.01 M PBS/ 5% NS/ 0.3% Triton X-100 (overnight, RT). Slides were washed in 0.01 M PBS (2 x 5 minutes, RT), followed by a quick rinse in dH₂O, and then incubated in 0.01 M

PBS/ 1% BSA/ 0.2% Triton X-100 (30 minutes, RT), with Cy3- conjugated AffiniPure donkey- anti- guinea pig or anti- goat IgG (1:1000; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody. Slides were washed in 0.01 M PBS (2 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-Microtubule-Associated Protein 2

Brain sections (15) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% NS/ 0.3% Triton X-100 (30 minutes, RT), and incubated with the primary antibody (microtubule-associated protein 2 (MAP-2) (1:500, Sigma)) in 0.01 M PBS/ 5% NS/ 0.3% Triton X-100 (overnight, RT). Slides were washed in 0.01 M PBS (2 x 5 minutes, RT), followed by a quick rinse in dH₂O, and then incubated in 0.01 M PBS/ 1% BSA/ 0.2% Triton X-100 (30 minutes, RT), with Cy3- conjugated AffiniPure goat anti- mouse IgG (1:1000; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody. Slides were washed in 0.01 M PBS (2 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-Neuronal Nuclei

Brain sections (20) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 5% NS/ 0.3% Triton X-100 (15 minutes, RT), and incubated with primary antibody (neuronal nuclei (NeuN) (1:300, Chemicon)), in 0.01 M PBS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS, then incubated in 0.01 M

PBS/ 0.2% Triton X-100 with Alexa Fluor 488 goat anti -mouse IgG (1:100; Invitrogen) secondary antibody (30 minutes, RT). Slides were washed in 0.01 M PBS (2 x 10 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-Neurofilament-70

Brain sections (15) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% NS/ 0.2% Triton X-100 (30 minutes, RT), and incubated with the primary antibody (neurofilament-70 (NF-70) (1:100, Chemicon)) in 0.01 M PBS/ 2% BSA/ 2% NS/ 0.1% Triton X-100 (overnight, 4°C). Slides were washed in 0.01 M PBS/0.2% Triton X-100 (2 x 5 minutes, RT), blocked in 0.01 M PBS/ 10% NS/ 0.2% Triton X-100 (30 minutes, RT), and then incubated in 0.01 M PBS/ 0.2% Triton X-100 (30 minutes, RT), with Fluorescein (FITC)-conjugated AffiniPure goat- anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody. Slides were washed in 0.01 M PBS/ 0.2% Triton X-100 (2 x 5 minutes, RT), followed by a wash in 0.01 M PBS (2 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-βIII Tubulin

Brain sections (15) were first fixed in 4% paraformaldehyde (10 minutes, RT), blocked in 0.01 M PBS/ 10% NS/ 0.1% Triton X-100 (30 minutes, RT), and incubated with the primary antibody (Anti-βIII Tubulin (TUJ1) (1:1000, Covance Research Products, Inc, Emeryville, CA; (1:200), Promega, Madison, WI; or

(1:100), Sigma) in 0.01 M PBS/ 5% NS/ 0.3% Triton X-100 (overnight, RT).

Slides were washed in 0.01 M PBS (2 x 5minutes, RT) followed by a quick rinse in dH₂O and then incubated in 0.01 M PBS/ 0.2% Triton X-100 (30 minutes, RT), with Fluorescein (FITC)-conjugated AffiniPure goat- anti- mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody. Slides were washed in 0.01 M PBS (3 x 5 minutes, RT) and coverslipped using VECTASHIELD[®] Mounting Medium with DAPI (Vector Laboratories).

Glial markers

Table3. Antibodies Used To Identify Glial Cells.

Glial Markers	Concentration/ Description	Company	Labels
Anti-Neuron Cell Surface Antigen, A2B5	1:300 Mouse monoclonal	Chemicon	Recognizes a cell surface ganglioside epitope expressed in developing thymic epithelial cells, oligodendrocyte progenitors, and neuroendocrine cells. Used to stain type II astrocytes, cells involved in gliogenesis, and cells committed to the oligodendrocyte lineage. Cytoplasmic label
Anti-Aspartoacylase	Rabbit polyclonal	Gift from Dr. Namboodiri	Predominantly stains oligodendrocytes Nuclear and cytoplasmic label.
2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase	1:1000 Rabbit polyclonal	Gift from Dr. Jacobowitz	Expressed at high levels by oligodendrocytes in the CNS and is absent in other cell types. Detects developing and adult myelin, developing oligodendrocytes and distinguishes oligodendrocytes from astrocytes, microglia, and neurons. Cytoplasmic.
Anti-Glial Fibrillary Acid Protein	1:200 Rabbit polyclonal	Dako	Class III intermediate filament protein that is heavily, and specifically, expressed in astrocytes and certain other astroglia in the CNS. Cytoplasmic label.
	1:1000 Mouse monoclonal	Gift from Dr. Jacobowitz	
Anti-Oligodendrocyte Transcription Factor 2	1:200 Rabbit polyclonal	Chemicon	Required for oligodendrocyte and motor neuron specification in the spinal cord, as well as for the development of somatic motor neurons in the hindbrain. Nuclear and cytoplasmic label.
Anti-S-100 β Chain	1:100 Goat polyclonal	Santa Cruz	Involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. This protein may function in neurite extension, proliferation of melanoma cells, stimulation of Ca^{2+} fluxes, inhibition of PKC mediated phosphorylation, astrocytosis and axonal proliferation, and inhibition of microtubule assembly. Nuclear and cytoplasmic label.
Anti-Vimentin	1:500 Mouse monoclonal	Abcam	Class III intermediate filament protein found in various non-epithelial cells including mesenchymal cells and fibroblasts. Cytoplasmic label.
	1:100 Mouse monoclonal	Chemicon	

Anti-A2B5

Brain sections (15) were fixed in 4% paraformaldehyde (15 minutes, RT), blocked in 0.01 M PBS/ 10% NS/ 0.3% Triton X-100 (30 minutes, RT), washed in 0.01 M PBS (2 x 5 minutes, RT), and incubated in the primary antibody (A2B5 (1:300; Chemicon,)), in 0.01 M PBS/ 0.2% Triton X-100 (overnight, RT). Slides were washed in 0.01 M PBS (2 x 5 minutes, RT), and incubated in 0.01 M PBS/ 0.2% Triton X-100, with the appropriate FITC-conjugated AffiniPure donkey anti - mouse IgM (1:200; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody (45 minutes, RT). Slides were washed in 0.01 M PBS (2 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti- Aspartoacylase

Brain sections (25) were rinsed in 0.01 M PBS, and incubated with the primary antibody (Aspartoacylase (ASPA) (1:1000, gift from Dr. Namboodiri)) in 0.01 M PBS/ 2% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 0.2% Triton X-100 (2 x 5 minutes, RT), and incubated in 0.01 M PBS/ 0.2% Triton X-100, with Alexa Fluor 488 goat- anti-rabbit IgG (1:100; Invitrogen) secondary antibody (1 hour, RT). Slides were washed in 0.01 M PBS/0.2% Triton X-100 (2 x 5 minutes, RT), then 0.01 M PBS (1 x 5 minutes, RT), and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase

Brain sections (15) were rinsed in 0.01 M PBS, and incubated with the primary antibody 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNP) [149, 150] (1:1000), gift from Dr. Jacobowitz) in 0.01 M PBS/ 2% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 0.2% Triton X-100 (2 x 5 minutes, RT) and incubated in 0.01 M PBS/ 0.2% Triton X-100 (1 hour, RT), with Alexa Fluor 488 goat- anti-rabbit IgG (1:100; Invitrogen) secondary antibody. Slides were washed in 0.01 M PBS/0.2% Triton X-100 (2 x 5 minutes, RT), then 0.01 M PBS (1 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-Glial Fibrillary Acid Protein

Brain sections (20) were rinsed in 0.01 M PBS, and incubated with the primary antibody (glial fibrillary acidic protein (GFAP) (1:200 DAKO Cooperation, Carpinteria, CA; or 1:1000 gift from Dr Jacobowitz) in 0.01 M PBS/ 10% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 1% BSA 3 x 5 minutes, and incubated in 0.01 M PBS/ 1% BSA/ 0.1% Triton X-100, with FITC-conjugated AffiniPure goat- anti- rabbit IgG (1:200) or Cy3- conjugated AffiniPure goat- anti- mouse IgG secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, Inc.) (30 minutes, RT). Slides were washed in 0.01 M PBS (2 x 5 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti- Oligodendrocyte Transcription Factor 2

Brain sections (15) were washed in 0.01 M PBS (3 x 3 minutes, RT), blocked in 0.01 M PBS/ 0.4% Triton X-100/ 1% BSA/ 25% NS (30 minutes, RT), and incubated with the primary antibody (Rabbit anti- Oligodendrocyte transcription factor 2 (Olig-2) polyclonal antibody (1:200 Chemicon)) in 0.01 M PBS/ 3% BSA (overnight, 4 °C). Slides were washed in 0.01 M PBS (3 x 3 minutes, RT), and incubated in 0.01 M PBS/ 3% BSA, with FITC-conjugated AffiniPure goat- anti- rabbit (1:100) or Cy3- conjugated AffiniPure goat- anti- rabbit secondary antibody IgG (1:1000; Jackson ImmunoResearch Laboratories, Inc.) (1 hour, RT). Slides were washed in 0.01 M PBS (3 x 3 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-S-100 β Chain

Brain sections (15) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 10% NS/ 0.3% Triton X-100 (15 minutes, RT), and incubated with the primary antibody (S-100 β chain (1:100, Santa Cruz Biotechnology Inc.) in 0.01 M PBS/ 5% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 0.2% Triton X-100, (4 x 5 minutes, RT) and then incubated in 0.01 M PBS/ 0.2% Triton X-100, with FITC-conjugated AffiniPure donkey- anti- goat secondary antibody IgG (1:200; 30 minutes, RT). Slides were washed in 0.01 M PBS (2 x 10 minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories).

Anti-Vimentin

Two protocols were utilized for this antibody. Procedure 1: Brain sections (10) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 1% BSA/ 10% NS (30 minutes, RT), and incubated in the primary antibody (Vimentin (1:500, Abcam) in 0.01 M PBS/ 0.3% Triton X-100/ 10% NS (overnight, RT). Slides were washed in 0.01 M PBS/ 1% BSA, (3 x 5minutes, RT) and then incubated in 0.01 M PBS/ 1% BSA/ 0.1% Triton X-100, with Cy3- conjugated AffiniPure goat- anti- mouse IgG or Fluorescein (FITC)-conjugated AffiniPure goat- anti- mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody (30 minutes, RT). Slides were washed in 0.01 M PBS (2 x 10 minutes, RT) and coverslipped using VECTASHIELD[®] Mounting Medium with DAPI (Vector Laboratories,).

Procedure 2: Brain sections (10) were rinsed in 0.01 M PBS, blocked in 0.01 M PBS/ 0.1% BSA/ 10% NS/ 0.1% Triton X-100 (15 minutes, RT), and incubated in the primary antibody (Vimentin (1:100, Chemicon) in 0.01 M PBS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS/ 0.2% Triton X-100, (2 x 5minutes, RT) and then incubated in 0.01 M PBS/ 0.2% Triton X-100, with Cy3- conjugated AffiniPure goat- anti- mouse IgG or FITC- conjugated AffiniPure goat- anti- mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody (30 minutes, RT). Slides were washed in 0.01 M PBS (2 x 10 minutes, RT) and coverslipped using VECTASHIELD[®] Mounting Medium with DAPI (Vector Laboratories).

Double Label Fluorescent Immunohistochemistry

Double label immunohistochemistry was performed to determine if any of the antibodies that gave a positive label with single label fluorescent immunohistochemistry also co-localized with BrdU in the goldfish brain. The antibodies used in conjunction with the antibody against BrdU included: H3, HuC/D, NeuN, ASPA, GFAP, and Vimentin. (See pages 26, 32, and 36 for Antibodies Tables) Antibodies that did not work for single label immunohistochemistry were not used for double label immunohistochemistry.

Two protocols were used. Protocol 1: Slides were rinsed in 0.01 M PBS, and DNA was denatured by incubating in 0.1 N HCl (5 minutes, 37 °C). The acid was neutralized in 0.1M borate buffer, pH 8.5 (5 minutes, RT) and slides were washed in 0.01 M PBS, (3 x 3 minutes, RT). Slides were blocked in 0.01 M PBS/ 5% NS/ 0.3% Triton X-100 (15 minutes, RT), and then incubated with the BrdU antibody (1:100) combined with one of the primary antibodies that successfully labeled in the single label immunohistochemistry in 0.01 M PBS/ 0.3% Triton X-100 (overnight, 4 °C). The concentrations of these antibodies were the same as in the single label section). Slides were washed in 0.01 M PBS (1 x 5 minutes, RT) and then incubated in FITC-conjugated AffiniPure goat- anti –rat (1:100), or Cy3- conjugated AffiniPure goat- anti -rat (1:1000; Jackson ImmunoResearch Laboratories, Inc.) for BrdU and the corresponding secondary of the second antibody (see single label immunohistochemistry section, page 24) in 0.01 M PBS/ 0.2% Triton X-100 (30 minutes, RT), washed in 0.01 M PBS (2 x 10

minutes, RT) and coverslipped using VECTASHIELD® Mounting Medium with or without DAPI (Vector Laboratories).

Protocol 2: Slides were washed 0.1M PBS (3 x 10 minutes, RT). DNA was denatured by incubating in 0.1N HCl (5 min., 37 °C). The acid was neutralized in 0.1M borate buffer, pH 8.5 (5 minutes, RT), and slides were washed in 0.1M PBS (6 x 20 minutes, RT). Slides were blocked in 0.01 M PBS/ 3% NS/ 1% Gelatin/ 1% BSA/ 0.3% Triton X-100 (30 minutes, RT), and then incubated in primary antibody (BrdU, 1:100) in 0.01 M PBS/ 3% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.1 M PBS, (3 x 10 minutes, RT), blocked in 0.01 M PBS/ 3% NS/ 1% Gelatin/ 0.3% Triton X-100 (30 minutes, RT), then incubated in FITC-conjugated AffiniPure goat- anti –rat (1:200), or Cy3- conjugated AffiniPure goat- anti -rat (1:1000; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody in 0.01 M PBS/ 3% NS/ 1% Gelatin/ 0.3% Triton X-100 (90 minutes, RT). Slides were washed in 0.1M PBS (3 x 10 minutes, RT), washed in 50 mM Tris HCl (pH 8.0) (30 minutes, RT) washed in 0.01 M PBS/ 0.1% Triton X-100 (2 x 10 minutes, RT), washed in 0.01 M PBS (2 x 5 minutes, RT), and blocked (30 minutes, RT) in 0.01 M PBS/ 3% NS/ 1% Gelatin/ 0.3% Triton X-100. Next slides were incubated in the second primary antibody in 0.01 M PBS/ 3% NS/ 0.3% Triton X-100 (overnight, 4 °C). Slides were washed in 0.01 M PBS (3 x 10 minutes, RT) blocked (30 minutes, RT) in 0.01 M PBS/ 3% NS/ 1% Gelatin/ 0.3% Triton X-100, and incubated in the corresponding secondary antibody of the second antibody (see single label immunohistochemistry section, page 24) in 0.01 M PBS/ 3% NS/ 1% Gelatin/ 0.3% Triton X-100 (3 hours, RT). Slides were

rinsed in 0.01 M PBS 4 times, once in 0.1M PBS and coverslipped using VECTASHIELD[®] Mounting Medium with DAPI (Vector Laboratories).

Images of the double label fluorescent immunohistochemistry were digitally captured (10 X, 20 X, 40 X, 60 X, 63 X, and/ or 100 X) with a Sony DKC 5000 Catseye digital still camera connected to a Nikon Labophot fluorescent microscope. Adobe[®] Photoshop[®] 5.5 was used to label and print images.

EXPOSURE OF GOLDFISH TO TRANYLCPROMINE

Experimental Design for Tranylcypromine Exposure

A total of fifty-six goldfish were used in this experiment. Goldfish were randomly placed into eight groups of seven fish each, comprising short or long term treatment with saline (control) or short or long term treatment with TCP administration followed by euthanasia after 16 hours or 5 days after BrdU exposure. Short term exposure was defined as 5 consecutive days of injections of either TCP or saline. Long term exposure was defined as 21 consecutive days of injections of either TCP or saline. The control fish received (10 µL/ g) saline (PBS) injections i.p., and the TCP fish received 10 µg/ g TCP (Biomol International, LP, Plymouth Meeting, PA) i.p. for either 5 or 21 consecutive days. (See Table 4 and Figure 3) for experimental variables.)

Table 4. Experimental Design for Tranylcypromine or Saline Exposure.

Treatment	Short term exposure		Long term exposure	
	16 hours post BrdU Group 1	5 days post BrdU Group 2	16 hours post BrdU Group 3	5 days post BrdU Group 4
Saline (control) (10 μL/ g)	5 days injection (n=7)	5 days injection (n=6)*	21 days injection (n=4)*	21 days injection (n=4)*
TCP (10 μg/ g)	5 days injection (n=4)*	5 days injection (n=5)*	21 days injection (n=4)*	21 days injection (n=3)*

* Experiment began with n=7 per group but some animals died and the actual number of those completing the experimental protocol are listed.

BrdU Exposure

Upon completion of the saline or TCP injections, the goldfish were placed into 1 L of tank water containing 5 mM BrdU (Sigma), and swam freely for 16 hours. The 16 hour exposure to BrdU represented a saturation-label of BrdU; where all cycling (mitotic) cells underwent at least one S-phase during the incubation and incorporation of BrdU [146]. After 16 hours, goldfish were either euthanized by decapitation immediately following the BrdU treatment (Groups 1 and 3) or placed back into the tank for an additional five days before euthanasia (Groups 2 and 4).

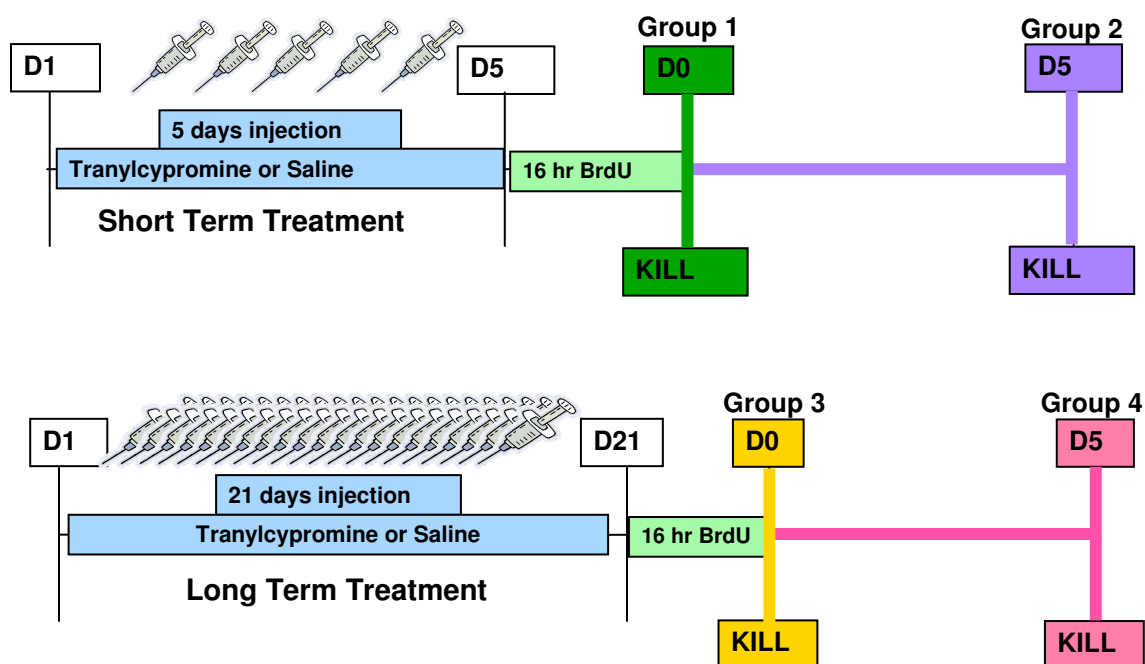


Figure 3. Schematic of treatment paradigm.

Euthanasia / Brain Harvesting

Fish were euthanized by decapitation (described under Euthanasia page 16). Brains were excised, placed onto a 1 cm x 2 cm copper wire mesh and rapidly frozen in an acetone-dry ice slurry (-87°C).

Sectioning

After euthanasia and tissue processing of the goldfish brain, tissue was sectioned on a Hacker-Bright OTF cryostat (Hacker Instruments Inc.) at $30\text{ }\mu\text{m}$. Serial sections were mounted on gelatin coated slides (as described under Sectioning on page 19).

Bromodeoxyuridine Peroxidase Immunohistochemistry

Sections were denatured by incubating the slides in 0.1 N HCl (2 minutes, 37 °C). The acid was neutralized in 0.1M borate buffer, pH 8.5 (2 minutes, RT). Endogenous peroxidases were blocked by incubating in 100% methanol/ 3% H₂O₂ (30 minutes, RT). Sections were blocked (30 minutes, RT) using the Vector Elite ABC Kit (Rat IgG) according to manufacturer (Vector Laboratories, Burlingame, CA). Briefly, three drops of the blocking serum provided in the kit was added to 10 mL of 0.01 M PBS. Slides were then incubated in the primary antibody monoclonal rat-anti BrdU (1:100, Accurate Chemical & Scientific Corporation) in 2% triton X-100/ 0.01 M PBS (overnight, RT). Slides were incubated in a biotinylated secondary rabbit anti rat antibody (Vector Elite Kit; three drops of the blocking serum plus one drop of the biotinylated secondary antibody provided in the kit was added to 10 mL of 0.01 M PBS) (30 minutes, RT) and rinsed in 0.1 M PBS (5 minutes, RT). Next, slides were incubated with Vectastain Elite ABC Reagent (two drops of Reagent A plus two drops of Reagent B were added to 5 mL of 0.01 M PBS, mixed and stood (30 minutes, RT) before use), (30 minutes, RT), followed by a rinse in 0.1M PBS (5 minutes, RT). The chromogenic development reagent, 3, 3' diaminobenzidine (DAB Substrate), (DAB substrate kit, Vector Laboratories) was then added and reacted until the desired brown to black insoluble precipitate developed. The DAB reaction was inhibited by placing slides into dH₂O (5 minutes, RT). Slides were then counterstained with Eosin.

Eosin Counterstaining

Following the completion of the DAB immunohistochemistry, tissue sections were counterstained with 1% Eosin (Sigma) in 80% ethanol for 30 seconds. The sections were dehydrated in increasing concentrations alcohol (80%, 95% ethanol (1 minute each), and in 100% ethanol (2 x 5 minutes)) [151]. Sections were then placed in xylene (2 x 1 minute) and coverslipped with Permount (Sigma).

Apoptotic Nuclear Profiles

The 16 hours post BrdU groups (control or TCP) were qualitatively assessed for apoptotic profiles. A collection of photomicrographs of the apoptotic-like nuclei were digitally captured (20 X, 40 X, 60 X, 63 X, and/ or 100 X) with a Sony DKC 5000 Catseye digital still camera connected to a Nikon biophot microscope. Adobe® Photoshop® 5.5 was used to label and print images.

Calculation of Number of Positively Labeled BrdU Cells

The results from the BrdU peroxidase immunohistochemistry were analyzed by examining sections at 20 X using a Nikon biophot microscope. For each goldfish brain, 30 μ m sections were serially collected extending from the frontal lobes through to the cerebellum. For the estimation of the total number of mitotic cells in a given brain region, the number of BrdU positive labeled cells were counted in every fifth section and multiplied by five.

Since the total number of BrdU positive cells counted in one section was multiplied by five, it was assumed that 5 adjacent sections had the same number of positive labeled cells. This calculation can lead to an overestimation of total BrdU labeled cell number unless that number is corrected for split nuclear errors. To correct for split nuclear errors, which would eliminate adding a nucleus twice when it extended between two adjacent sections, the resulting number ("X" times 5) was multiplied by a correction factor derived from the following formula developed by Abercrombie (1946) [152]:

$$N_i = (n_i) \cdot 5 \cdot [(t) \div (t + d)]$$

N_i = total number of BrdU positive cells in adjacent sections

where N_i is the number of BrdU- labeled cells present in the section "i" times 5; n_i is the number of labeled cells actually counted in section "i"; "t" is the section thickness; and "d" is the mean diameter of the BrdU labeling precipitate in a nucleus. To obtain the mean diameter of the BrdU labeling precipitate in a nucleus, one hundred positively labeled nuclei were measured and the average was of those values was applied to this formula.

An example of this formula:

$$N_i = (n_i) \cdot 5 \cdot [(t) \div (t + d)] =$$

$$N_i = (200 \text{ cells counted}) \cdot 5 \cdot [(30 \text{ } \mu\text{m thickness}) \div (30 \text{ } \mu\text{m thickness} + 7.5 \text{ } \mu\text{m mean diameter of BrdU positively labeled nuclei})] =$$

$$N_i = (200 \text{ cells}) \cdot 5 \cdot [(30 \mu\text{m}) \div (37.5 \mu\text{m})] =$$

$$N_i = (1000 \text{ cells}) \cdot [0.8] =$$

$$N_i = 800 \text{ cells}$$

Statistical Analysis

The data resulting from these calculations were analyzed by *Student's t*-test, or analysis of variance followed by Tukey post hoc test, using the software program GraphPad Prism (version 3.02) for Windows. Results are expressed as mean $\pm$ SEM.

CHAPTER 3

RESULTS

ADULT GOLDFISH BRAIN

The atlas of the adult goldfish brain is presented in Plates 1 to 30 (see Appendix for plates and abbreviations). Stereotaxic skull coordinates for the goldfish brain are presently unavailable because increased brain size alters the brain position within the cranial cavity. As goldfish increase in body size, so do their brains proportionately. In order to compensate for brain position changes, a formula would need to be developed [127]. As an alternative, identification of major divisions and landmarks of the brains was accomplished with the aid of a stereological microscope. In this atlas the rostral extent of the goldfish brain is the olfactory bulbs and the caudal extent is the cervical spinal cord.

Nomenclature

The goldfish brain atlas presented here contains the English nomenclature as translated from the Latin and where appropriate, can be correlated to the mammalian brain. The nomenclature adapted for this atlas is based on previous publications [58-60, 63, 65, 67, 101, 102, 126, 127, 129-131, 133-137, 139-143, 153]. Additionally, several recent reviews on teleost forebrain were also used to verify that the nomenclature conformed to recent standards. All descriptions were compared to previous morphological reports from various fishes: zebrafish [67, 102, 136, 137, 139], brown ghost knifefish [58-60, 65, 101, 140], carp [135],

three-spined fish [63], and others [126, 127, 129-131, 133-135, 141-143, 153].

Abbreviations for the nomenclature of the nuclei and fiber tracts are listed on page 116.

Forebrain: Telencephalon and Diencephalon

The forebrain consists of the telencephalon and diencephalon. The telencephalon is comprised of the olfactory bulbs (Plates 1 - 2) and frontal lobes (Plates 3 - 8).

The telencephalon is divided into the left and right hemisphere by the telencephalic ventricle. Histologically, each hemisphere is divided into the dorsal and ventral area of the telencephalon. The dorsal area of the telencephalon is generally referred to as the pallium while the ventral area of the telencephalon is referred to as the subpallium.

Both the dorsal and ventral areas of the telencephalon are histologically divided into additional distinct zones. Progressing caudally, the dorsal area of the telencephalon is subdivided in the dorsal medial (Dm), dorsal dorsal (Dd), dorsal ventral (Dv), dorsal central (Dc), and dorsal posterior (Dp) zones (Plates 3 - 8). Located in middle part of the telencephalon, the lateral forebrain nucleus (LFB) (Plate 6 - 7) was situated between the ventral aspect of the dorsal telencephalon and the dorsal aspect of the ventral telencephalon. The ventral area of the telencephalon was subdivided in the ventral dorsal (Vd), ventral ventral (Vv), ventral lateral (Vl), and ventral posterior (Vp) zones (Plates 3 - 7). Situated ventromedially and ventrolaterally are the medial and lateral olfactory

tracts (MOT, LOT respectively) (Plate 3 - 6). The entopeduncular nucleus (E) (Plate 5 – 8; 10 – 11) was located ventromedial to the LOT. Progressing caudally the E was positioned dorsomedial to the optic tract and dorsolateral to the thalamic nuclei in the diencephalon. In the middle portion of the telencephalon, the two hemispheres were connected by the anterior commissure (AC) (Plates 5 - 6).

The diencephalon is located caudal to the telencephalon (Plates 5 - 22). The optic tract (OT) and optic chiasm (OC) extend on the ventrolateral sides of the diencephalon (Plate 5 – 7; 9 - 10). The dorsomedial OT extended towards the optic tectum (OTe) while the ventrolateral OT was elongated more caudally.

The third ventricle appeared in the rostral portion of the diencephalon along the midline between the two hemispheres. The preoptic area, which includes the parvocellular preoptic nucleus, anterior part (PPa) (Plates 5 - 8), collectively surrounded the rostral part of the third ventricle. Progressing caudally, the third ventricle expanded transversely and divided into the lateral recesses (Plates 12 - 18). Most thalamic and hypothalamic nuclei were situated along these ventricles. The horizontal commissure (HC) (Plates 8 – 9) emerged ventrally in the rostral diencephalon. At the level of the HC, the anterior periventricular nucleus (APN) could be seen dorsomedial to the HC (Plate 9, 12). Ventrolaterally, the preglomerular complex (PG) appeared rostral to the nucleus diffusus of the inferior lobe (DF) (Plate 8).

The habenula (H) (Plate 9 - 10) surrounded the dorsal periventricular region of the third ventricle. The habenulointerpeduncular tract/ fasciculus retroflexus

(FR) (Plates 10 -13) was located ventral and caudal to the habenula. Also located caudally to the habenula were the thalamic nuclei situated periventricularly: ventromedial (Vm), ventrolateral (Vl), central posterior (CP), and dorsomedial nucleus of the thalamus (NDM) thalamic nuclei (Plate 9 - 10).

The periventricular nucleus of posterior tuberculum (TPp) was located caudal to the ventral thalamus and elongated caudally along third ventricle. Progressing ventrally the posterior periventricular nucleus (PPN), anterior tuberal nucleus (ATN), and lateral tuberal nucleus (NLT) were positioned periventricularly in a columnar pattern (Plates 10 - 12). Lateral to the PPN, the medial pretoral nucleus (MPN) could be found dorsomedial to the tertiary gustatory nucleus (TGN) (Plates 12 - 13).

In the ventral part of the diencephalon the ventral zone of periventricular hypothalamus (Hv), dorsal periventricular hypothalamus (Hd), caudal periventricular hypothalamus (Hc), and lateral hypothalamic nucleus (LH) were situated (Plates 12, 14 - 18). In the caudal hypothalamus, the mammillary bodies (MB) were observed at the midline (Plate 17).

Mesencephalon

The mesencephalon was characterized by the dorsally expanding optic tectum and the wide mesencephalic ventricle. The mesencephalon could be divided into three major subdivisions dorsal to ventral: the optic tectum (OTe) (Plates 9 – 22), torus semicircularis (TS) (Plates 16 – 22), and the tegmentum.

The posterior commissure (PC) separated the mesencephalon from the diencephalon.

The periventricular gray zone (PGZ) (Plates 10 - 22) formed the medial layer of the OTe. The OTe with the PGZ formed the lateral walls of the mesencephalic ventricle. Torus longitudinalis (TL) (Plates 11- 19) hung over the mesencephalic ventricle medially and extended rostrocaudally. Ventrocaudal to the mesencephalic ventricle, TS was positioned and as it progressed caudally, expanded into the mesencephalic ventricle.

Within the medial portion of the tegmentum was the origin of the medial longitudinal fascicle (MLF), which was also near the nucleus of the medial longitudinal fascicle (NMLF) (Plates 13 - 15). The lateral longitudinal fascicle/ lateral lemniscus (LL) began near TS and was ventral to the dorsal tegmental nucleus (DTN) (Plates 15 – 18). Both the MLF and the LL extended towards the rhombencephalon.

Rhombencephalon

The rhombencephalon, the most caudal brain (hindbrain) consisted of the meta- and myelencephalon. Since fish lack the pons, the metencephalon served as cerebellum and the myelencephalon served as the medulla oblongata [141].

The cerebellum is divided into 3 main regions longitudinally: the valvula cerebelli (V), the corpus cerebelli (CC), and the caudal cerebellar region. The valvula cerebelli protruded into the mesencephalic ventricle medially, and consists of a molecular and granular layer (ML and GL respectively). The

molecular layer was situated dorsal to the granular layer. Each layer consists of a medial and a lateral area (VMM, VML, VMG, VGL) (Plates 11 - 20).

Progressing caudally, the CC replaces the VML and VGL with a molecular and granular layer (CCML and CCGL) (Plates 19 - 24). The anterior cerebellar tract (ACT) (Plates 19 -21) forms beneath the CCGL. At approximately this level, the secondary gustatory nucleus (SGN) (Plates 19 - 20) and lateral valvula nucleus (NLV) (Plates 19 - 20) could be seen ventral to the ACT.

Caudally, the CC forms a single lobe. At this level, the granular eminence (GE) emerges (Plate 22) medially and spreads laterally and caudally. Surrounding the rhombencephalic ventricle, the granular layer of caudal lobe of cerebellum (LCAG) forms the roof while the cerebellar crest of the CC forms the floor of the ventricle at its rostral portion (Plates 22 - 23). The rhombencephalic ventricle or fourth ventricle was situated between the cerebellum and the medulla oblongata. This ventricle is narrow at the rostral part and eventually widens as it progresses caudally, with its greatest width found at the middle part of the medulla oblongata.

Situated in the ventral aspect of the cerebellum and ventral to CC was the medial octavolateralis nucleus (MON). Ventromedial to the MON was the sensory root of the facial nerve (VII_s) (Plate 23). The intermediate reticular formation (IMRF) (Plates 22 - 29) was positioned below the fourth ventricle.

The level where the cerebellum disappears coincides with the location for the appearance of the facial (LVII) and vagal lobes (LX) (Plates 25 - 29). The LVII was located centrally and the sensory root of the facial nerve (VII_s) (Plate

25) was just lateral and caudal to it. Progressing caudally, the LVII is surrounded by the central gray zone. The LX was positioned lateral to LVII and consists of three zones. From medial to lateral, these zones are the motor (MZ), fiber (FZ), and sensory (SZ) zones (Plates 25 -28). The motor nucleus of the vagal nerve (NXm) (Plates 25 -26) is found ventrally at midline and slightly lateral to the ventricle. Ventrolaterally, the vagal nerve (X) extends with the sensory (Xs) portion lateral to the motor (Xm) portion of the nerve (Plates 25 – 28).

At the most caudal area of the brain, the area postrema (AP) (Plate 29) was the most median structure. Ventral to the AP was the dorsal motor nucleus of the vagus nerve (DMNX) (Plate 29). Along the ventral midline, the MLF and IMRF are still present (Plates 21 – 29). The atlas ends with a section of the spinal cord with the dorsal horn (DH) dorsal to the ventral horn (VH) (Plate 30).

PROLIFERATION ZONES IN THE ADULT GOLDFISH BRAIN

Mapping and Identification of Proliferation Zones

The distribution of cells that had incorporated BrdU in three goldfish, obtained after continuous 24 hour BrdU incorporation, was used to identify proliferation zones throughout the brain of the adult goldfish. BrdU labeled cells were found throughout the brain from the frontal lobe to the vagal lobes.

Labeling with BrdU was consistent in the brains examined, both in intensity and localization. Examples of typical patterns of positive BrdU label within the optic tectum and rostral cerebellum are show in Figure 4.

A qualitative assessment of the distribution of the BrdU labeled cells in the brain of the three goldfish was done by plotting the cells onto their respective corresponding cresyl violet stained sections. The plots of the BrdU positive cells found in all three brains were merged (Figure 5.) In some cases, each dot represented one cell. However, due to the density of the BrdU labeled cells in some areas, the region of interest with the number of positive cells was noted instead of each individual cell. The resulting plots were merged onto the appropriate image from the atlas of the adult goldfish brain to determine the location of proliferation zones throughout the brain (Figure 6, Appendix II).

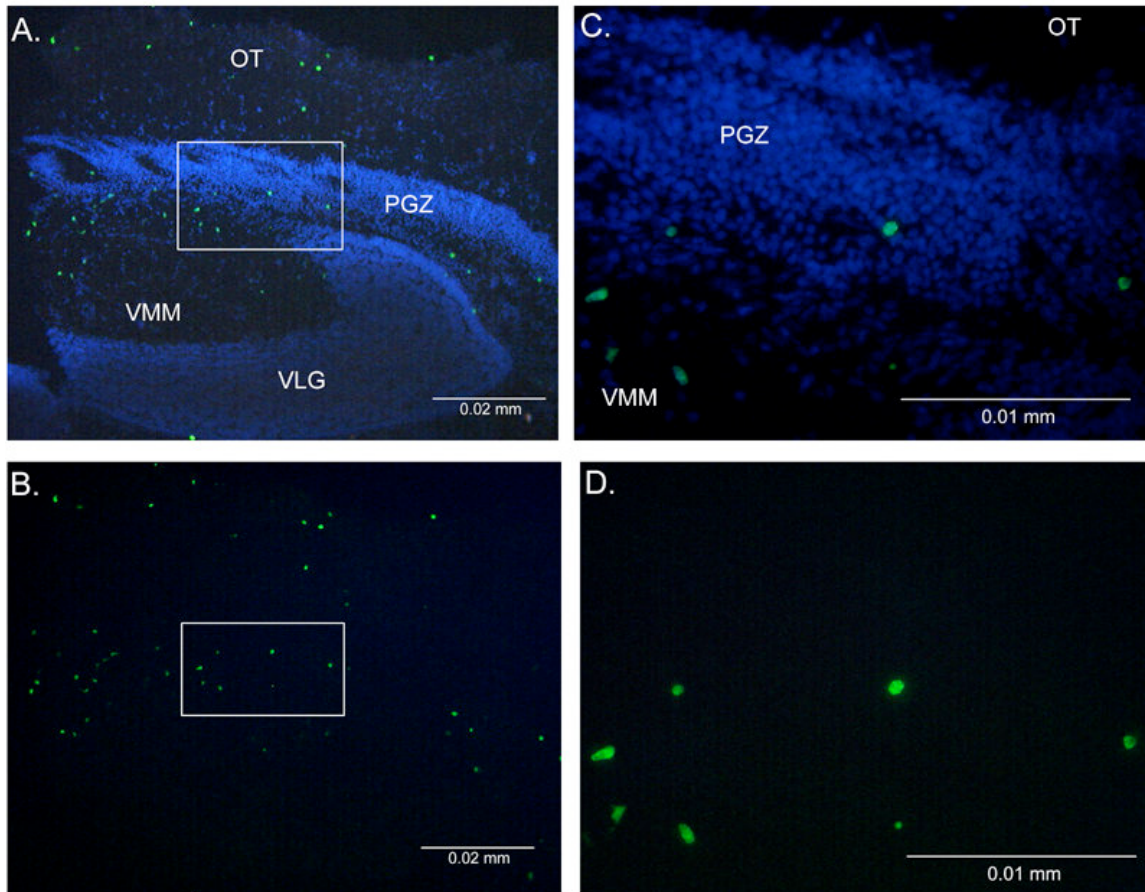


Figure 4. BrdU fluorescent labeling in the optic tectum and cerebellum. Normal fish were killed 24 hours after continuous BrdU administration. A. and C. are merged images of BrdU (green) and DAPI nuclear label (blue). B. and D. are of BrdU only. A,B. Low magnification of BrdU positive cells in the optic tectum and cerebellum. C. High magnification of BrdU positive cells from the boxed region of A. D. High magnification of BrdU positive cells from the boxed region of B.

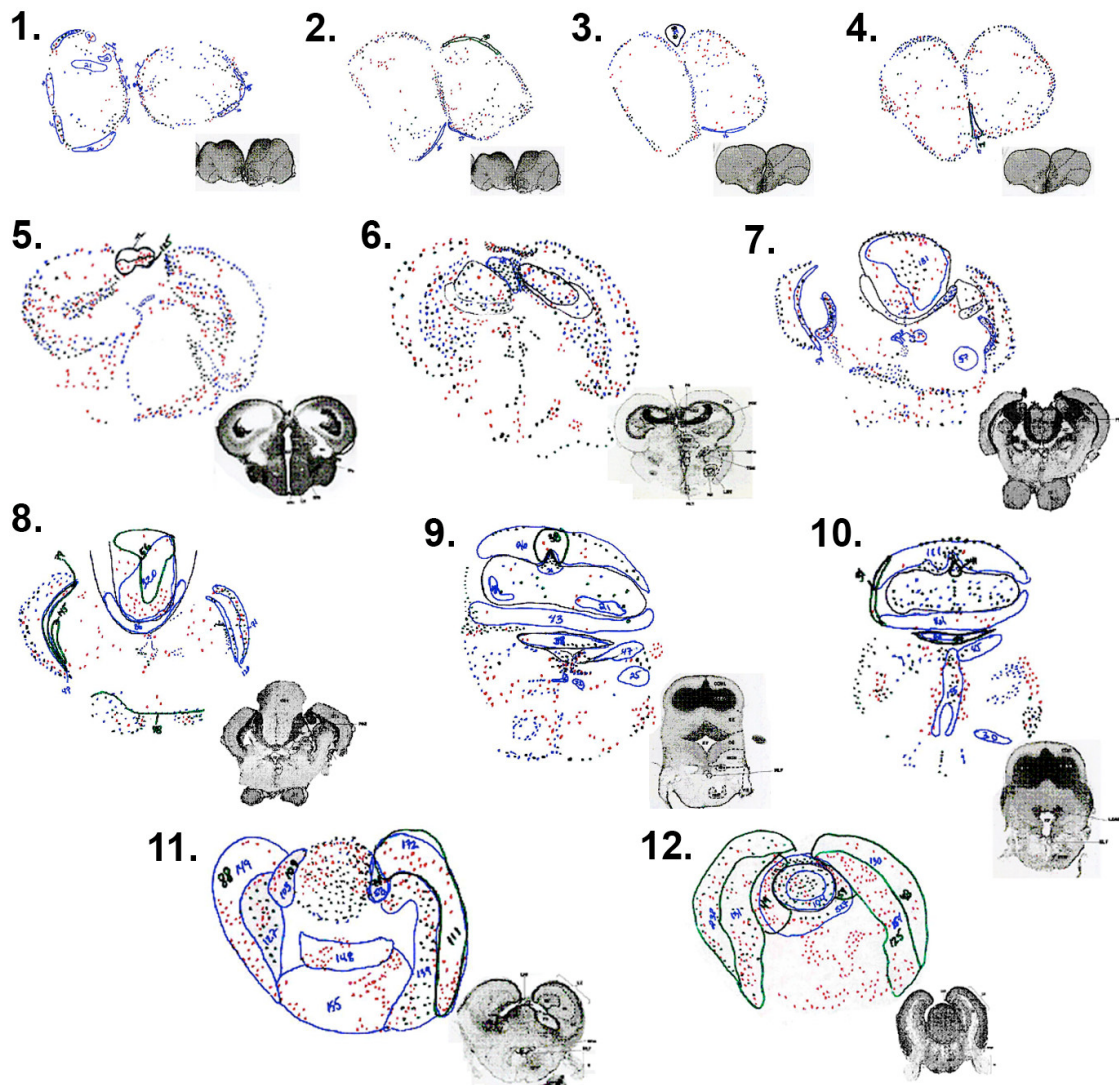
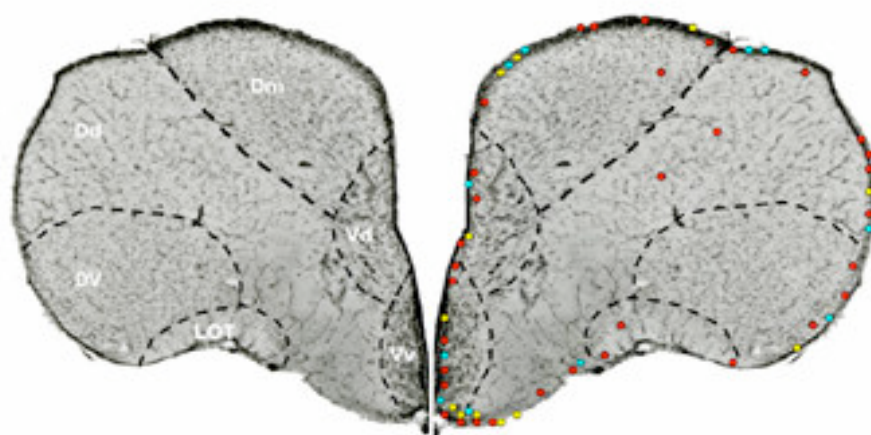
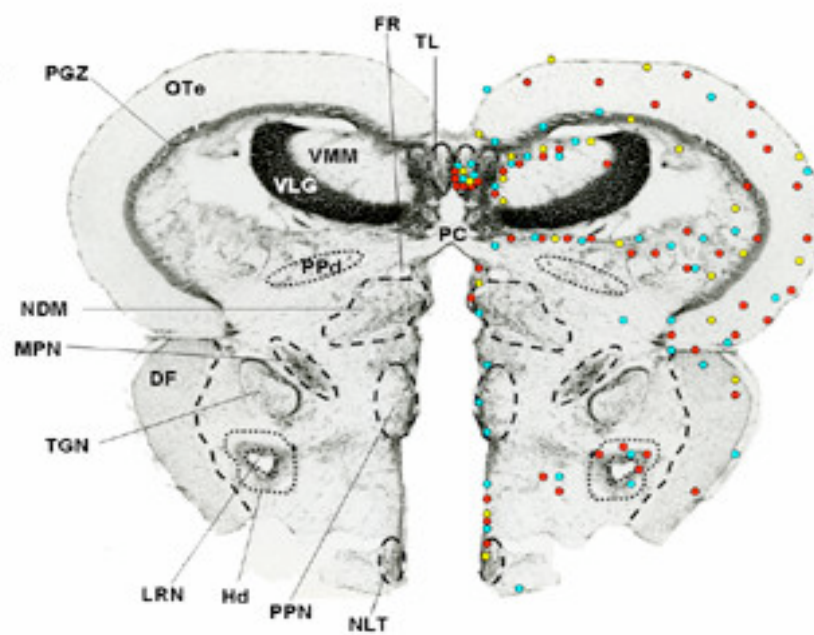
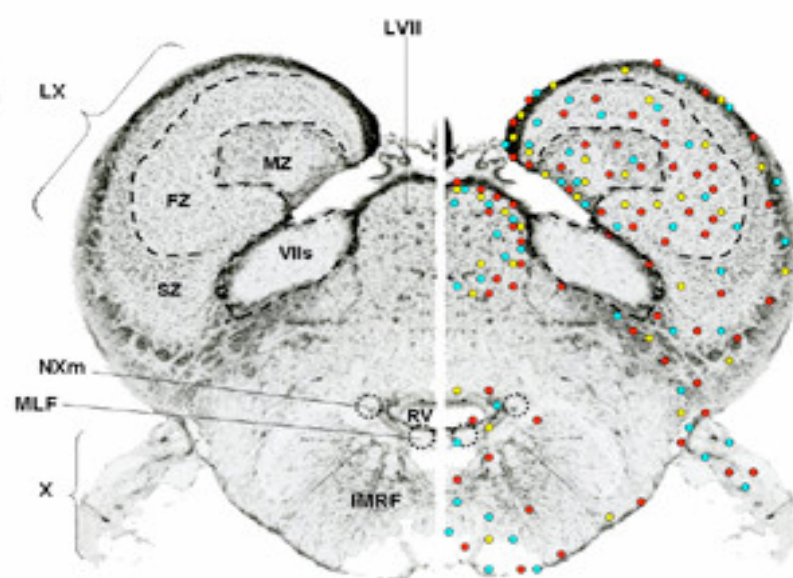


Figure 5. Composite of BrdU labeled cells in the adult goldfish brain. Goldfish were killed 24 hours after continuous BrdU administration. The brain sections begin rostral (1.) and progress caudally in numerical order. Each circle color (green, blue and red) represent BrdU labeling from one fish. Each dot represented one BrdU labeled cell. For areas dense with BrdU labeled cells, the region of interest with the number of positive cells was noted instead of each individual cell. These cells were plotted onto corresponding sections from the adult goldfish atlas.

Figure 6. Location of BrdU positive cells in the adult goldfish brain. Goldfish were killed 24 hours after continuous BrdU administration. Each circle color (red, yellow or blue) represents BrdU positive immunofluorescent label from one fish. Each dot represents the relative densities (~ 1-5 cells) of positive BrdU labeled cells. BrdU positive cells were mapped onto corresponding sections from the atlas of the adult goldfish based on cresyl violet stained sections. BrdU positive cells are indicated in the: telencephalon (A); optic tectum, cerebellum, and hypothalamus (B); and facial and vagal lobes (C).

A.**B.****C.**

Telencephalon

The vast majority of BrdU positive cells were concentrated at the midline where the telencephalic ventricle lies between the left and right lobes of the telencephalon (Figures 5, 6A, 7). Labeled cells were restricted to a narrow zone that extended mediolaterally in the dorsal, medial, ventral, lateral, and posterior zones of the dorsal telencephalon. In the ventral telencephalon, the BrdU labeled cells were restricted to a narrow zone that extended along the periphery of the dorsal, lateral and ventral zones of the ventral telencephalon.

Mesencephalon

BrdU positive cells were found along the ventral aspect of torus longitudinalis (TL), the area adjacent to the mesencephalic ventricle. Labeled cells were also detected at the lateral borders where TL and the dorsal portion of the optic tectum convened. The optic tectum generally contained BrdU labeled cells within its superficial layers and these cells were homogenously distributed (Figures 4B, 5, 8C). In general, the periventricular gray zone had a homogeneous distribution of BrdU positive cells throughout the majority of its rostrocaudal extent (Figures 5, 6B, 8D). At the caudal end of this structure, labeled nuclei were densely clustered in a constricted segment at the medial boundary and the cerebellar lobe, separated by the caudal extent of the mesencephalic ventricular space.

Cerebellum

The cerebellum is divided into rostral and caudal regions encompassing the valvula cerebellum and the corpus cerebellum respectively. BrdU labeled cells

were found in the molecular layers of the medial and lateral valvula cerebellum (Figures 5, 6B). In the corpus cerebellum, BrdU positive cells were found in the dorsal molecular layer (Figure 9C). They were positioned ventral to the pial surface in a concentrated band. BrdU labeled cells were also detected along the midline in the molecular layer of the corpus cerebellum along the ventrodorsal axis (Figure 9D).

Rhombencephalon

BrdU labeled cells were found in the facial and vagal lobes of the hindbrain. Positive cells were dispersed throughout both lobes (Figures 5, 6C, 10). In the facial lobe, positive cells congregated along the periphery near the fourth ventricle while the central gray zone contained numerous scattered BrdU positive cells. The vagal lobe contained labeled cells dispersed throughout the sensory, fiber and motor zones. Within the reticular formation, labeled cells were homogenously dispersed.

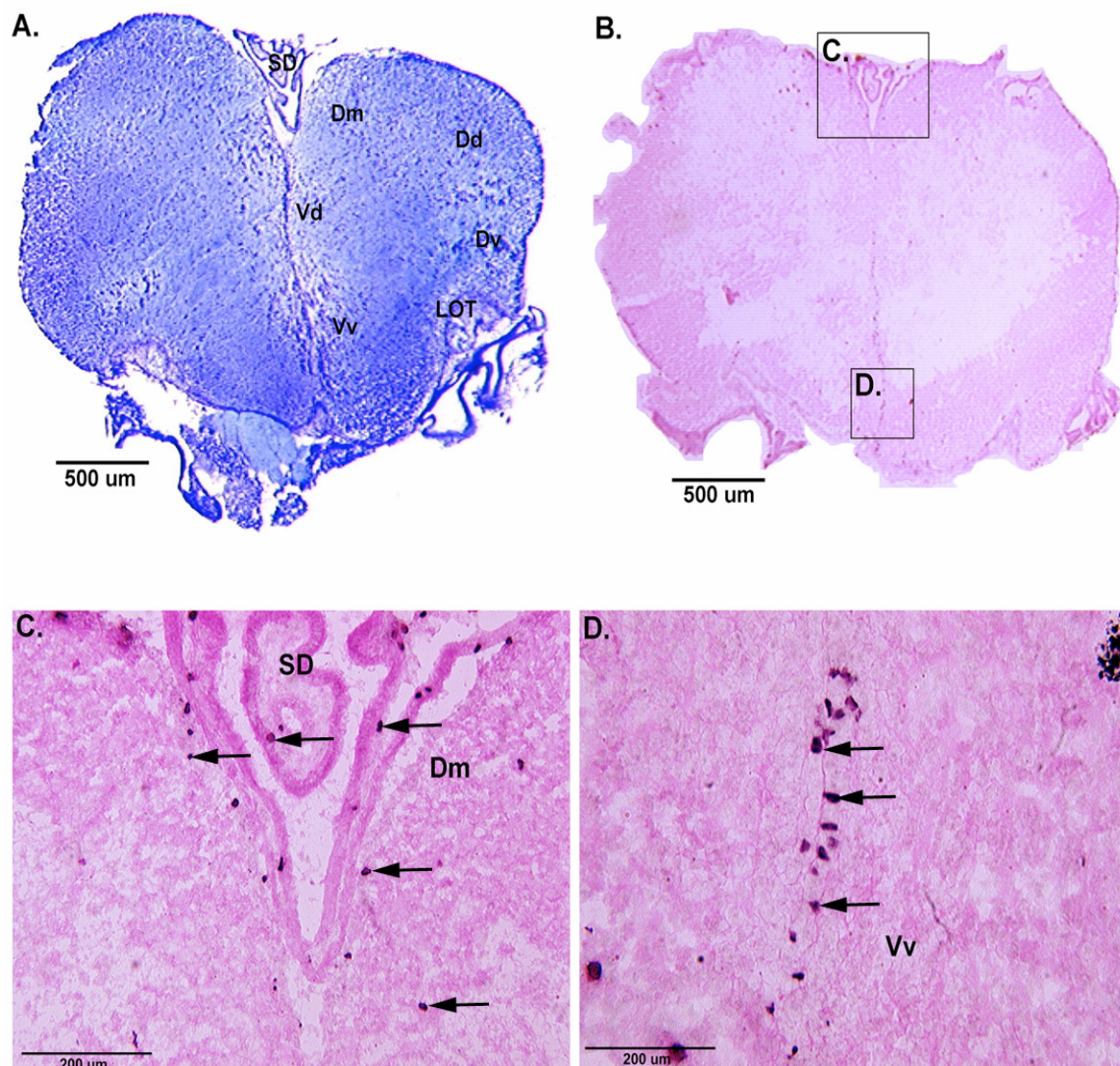


Figure 7. Proliferation zones in the rostral telencephalon of an adult goldfish. Examination of a coronal section after 16 hours administration of continuous BrdU revealed mitotic cells located at or near ventricular surfaces. A. Cresyl violet stained section from the telencephalon. Dd, dorsal zone of dorsal telencephalic area; Dl, lateral zone of dorsal telencephalic area; Dm, medial zone of dorsal telencephalic area; Dv, ventral zone of the dorsal telencephalic area; LOT, lateral olfactory tract; SD, saccus dorsalis; Vd, dorsal zone of the ventral telencephalic area; and Vv, ventral zone of ventral telencephalic area. B. Adjacent low magnification of the telencephalon processed by BrdU DAB peroxidase immunohistochemistry and counterstained with eosin. Boxes indicate regions enlarged in C and D. C. Region of the SD and Dm at higher magnification. BrdU labeled cells are depicted by arrows. D. Region of Vv at higher magnification. BrdU labeled cells are indicated by arrows.

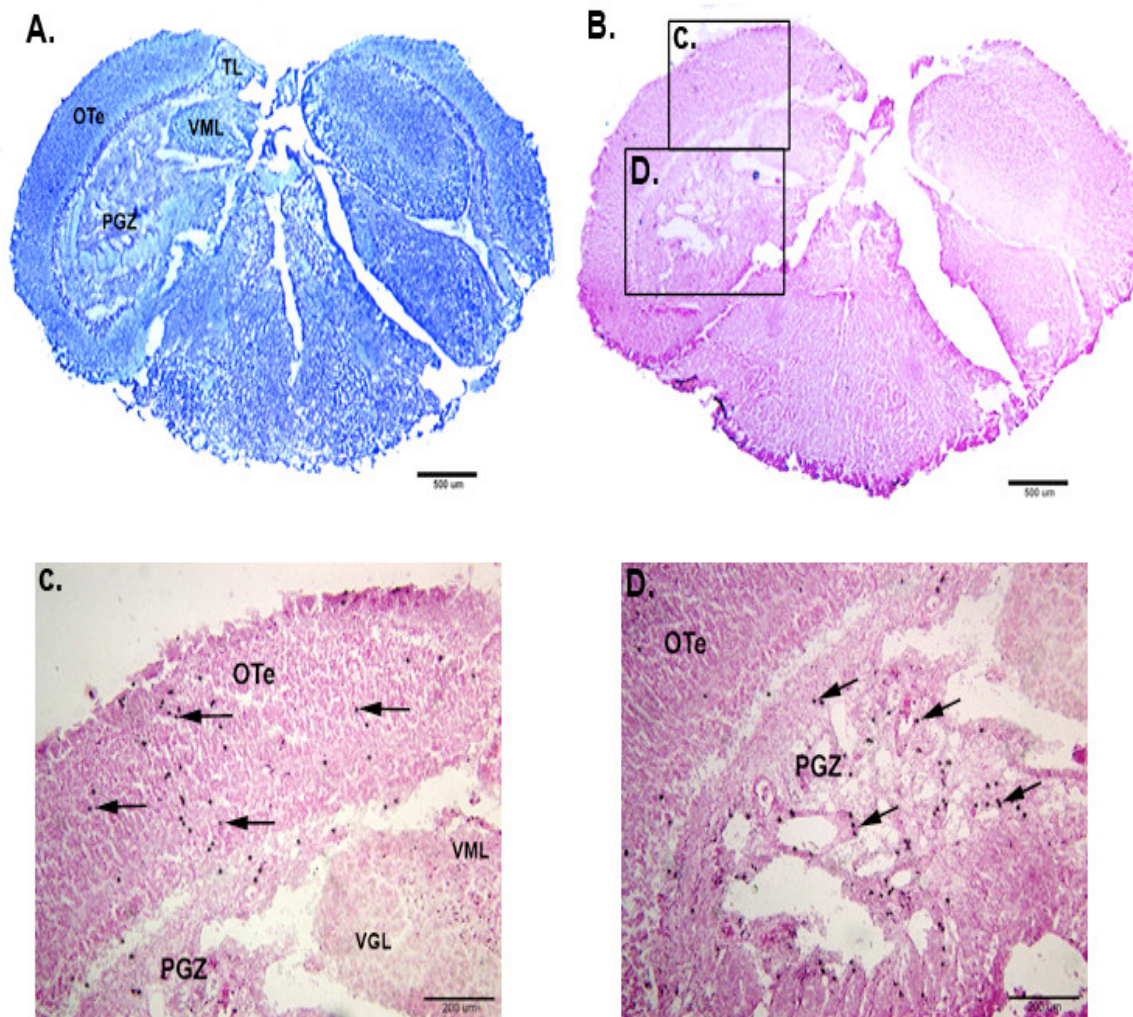


Figure 8. BrdU labeled cells in a coronal section of the optic tectum after 16 hours administration of continuous BrdU. Mitotic cells were located at or near ventricular surfaces. A. Cresyl violet stained section. OTe, optic tectum; PGZ, periventricular gray zone; TL, torus longitudinalis; VML, molecular layer of the valvula cerebellum. B. Adjacent low magnification of the optic tectum processed by BrdU DAB peroxidase immunohistochemistry and counterstained with eosin. Boxed areas are enlarged regions in C and D. C. Optic tectum at higher magnification showing BrdU labeled cells (arrows). VGL, granular layer of the valvula cerebellum. D. PGZ at higher magnification showing BrdU labeled cells (arrows).

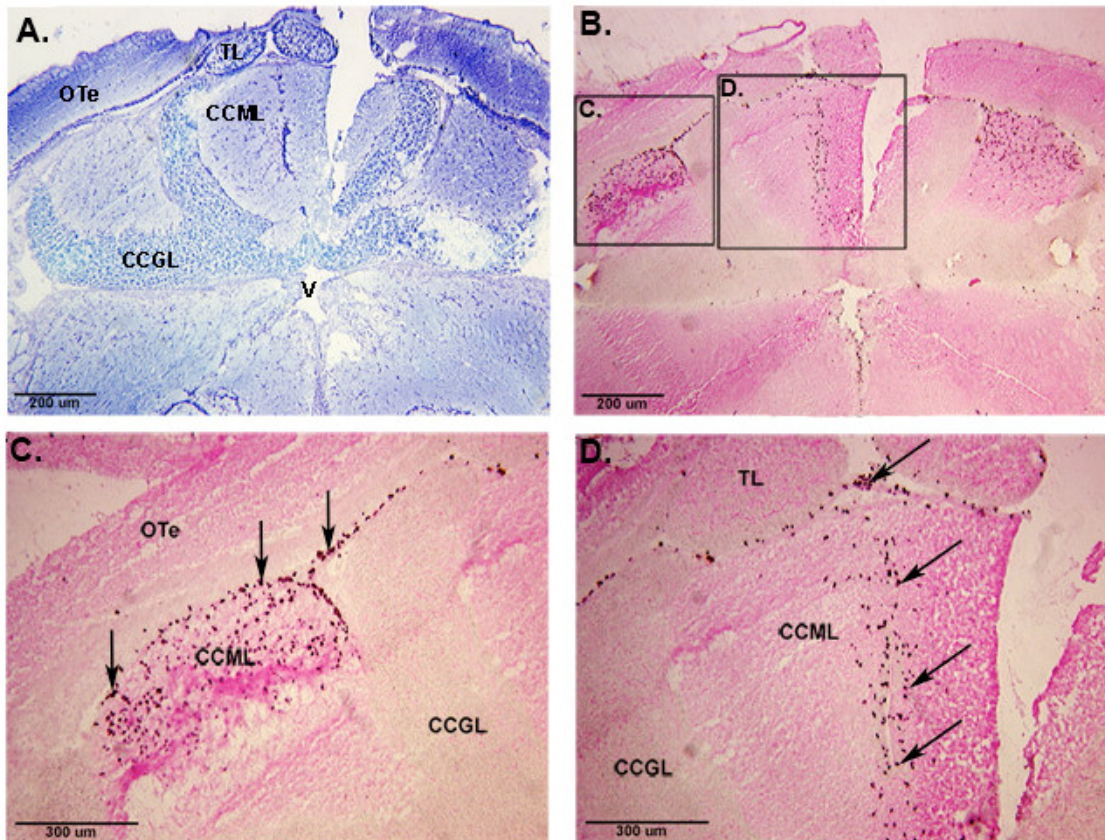


Figure 9. BrdU labeled cells in a coronal section of the cerebellum after 16 hours administration of continuous BrdU. As seen in other brain regions, mitotic cells were located in the molecular layers near the ventricular surfaces. A. Cresyl violet stained section. CCGL, granular layer of the corpus cerebellum; CCML, molecular layer of the corpus cerebellum; OTe, optic tectum; TL, torus longitudinalis; V, ventricle. B. Adjacent low magnification of the medial cerebellum processed for BrdU DAB peroxidase immunohistochemistry and counterstained with eosin. Boxed areas are enlarged regions in C and D. C. Region of the corpus cerebellum at higher magnification. BrdU labeled cells (arrows) were located within the CCML and at ventricular surfaces between the cerebellum and optic tectum. D. Region of the valvula cerebellum at higher magnification. BrdU labeled cells (arrows) located within the molecular layer of the valvular cerebellum (VML).

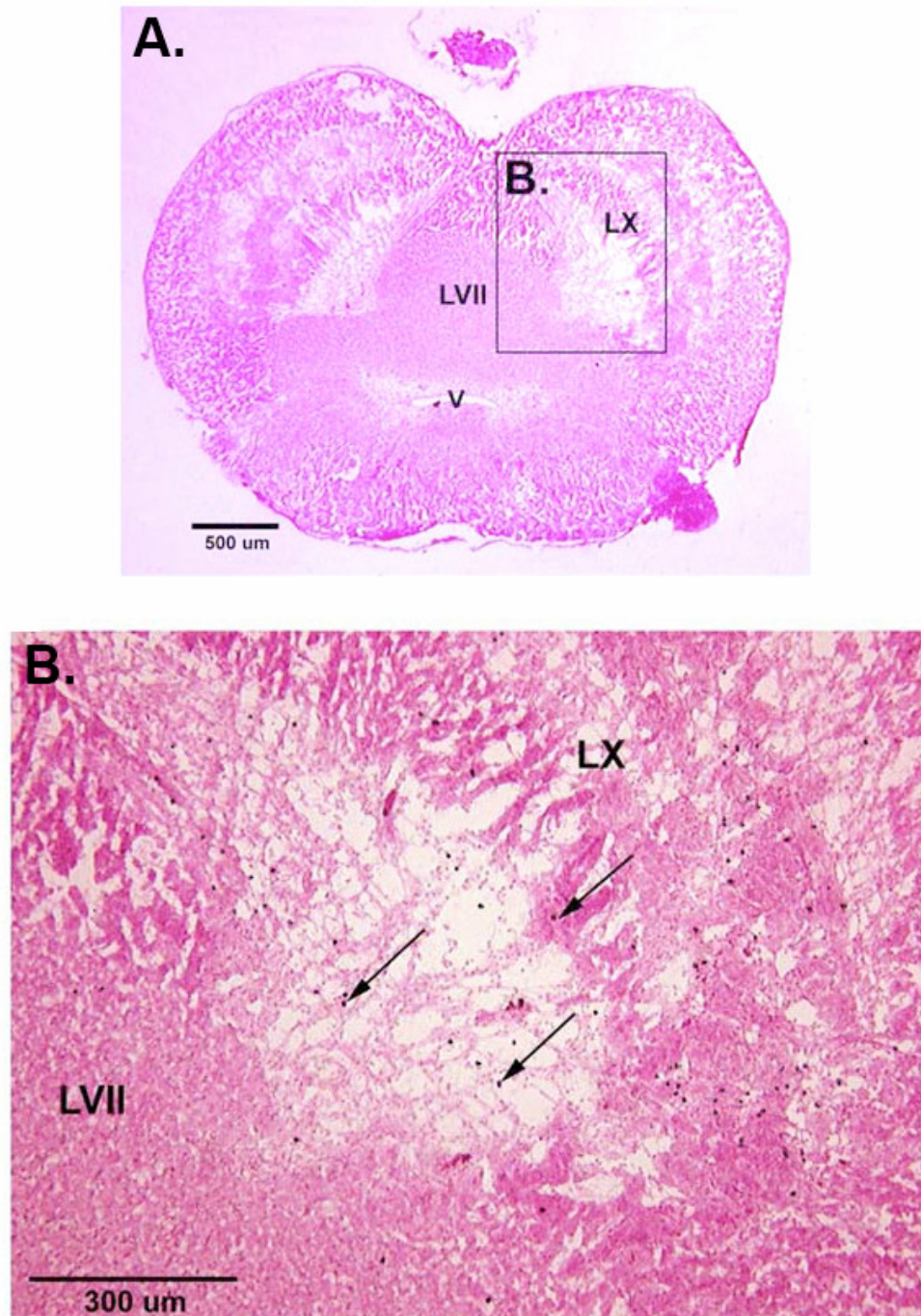


Figure 10. BrdU labeled cells in the vagal lobe after 16 hours of continuous BrdU. A. Low magnification of the vagal (LX) and facial (LVII) lobes processed for BrdU DAB peroxidase immunohistochemistry and counterstained with eosin. Boxed areas enlarged in B. V, ventricle. B. Region of the central gray zone between the vagal and facial lobes at higher magnification. BrdU labeled cells are denoted by arrows and located near the ventricle.

Immunohistochemical Co-localization of Mitotic Markers

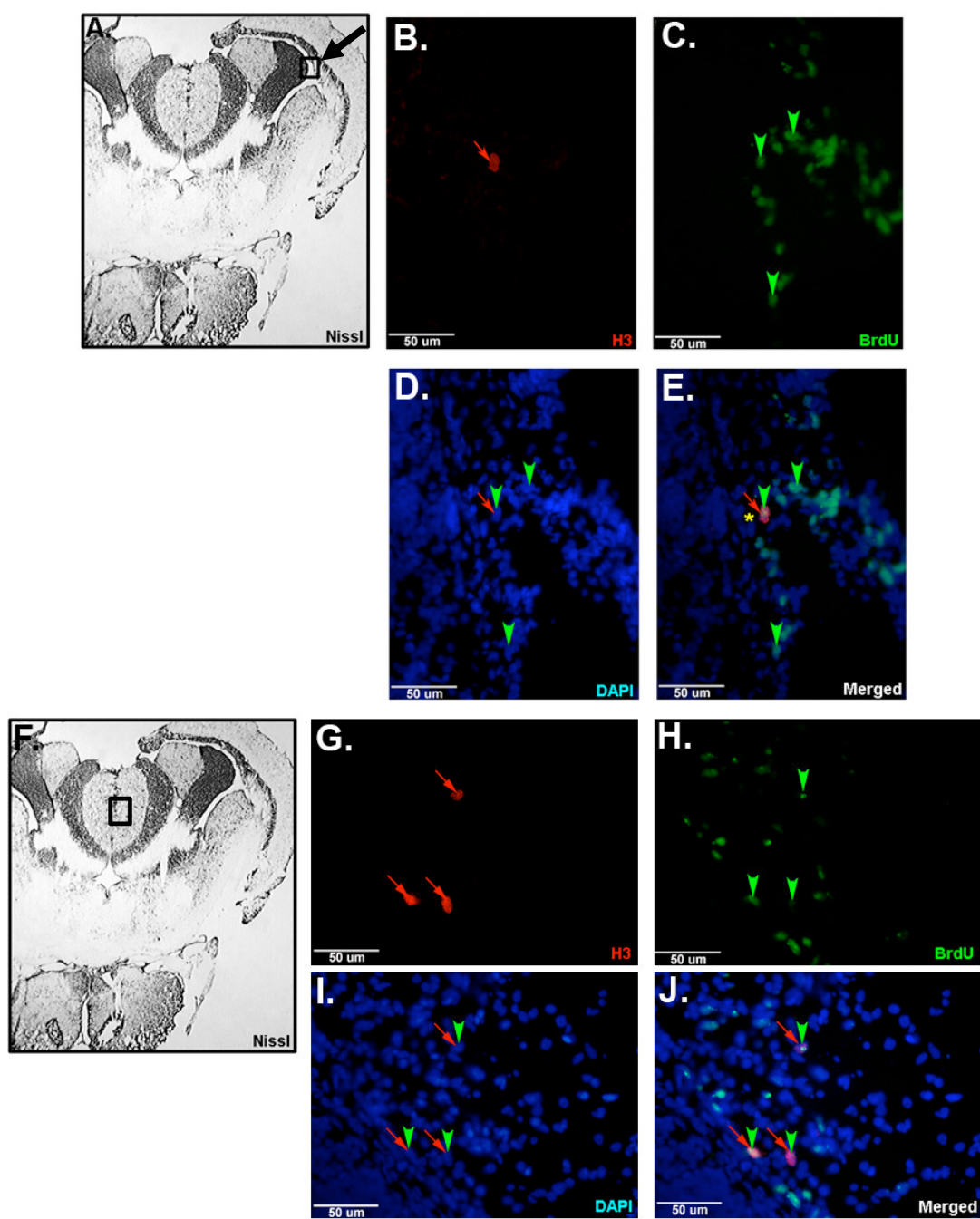
To confirm the BrdU labeling, other known mitotic antibody markers, including PCNA, Ki67, Pax-6, and H3, were used to show evidence of cell division. Only the H3 had positive results, and was found to co-localize with BrdU (Figure 11). The other antibodies used did not work due to technical difficulties. H3 staining was found to overlap regions similar to BrdU, but at a lower density (Figure 11). The difference between the labeling of these two mitotic markers was that BrdU was administered continuously for 24 hours and was thus incorporated into the newly synthesized DNA of all replicating cells during that time period. In contrast, H3 was correlated to cells that were undergoing chromosome condensation at the specific time-point when the animal was euthanized.

Differentiation Pattern of Post Mitotic Cells

To determine the identity of cells that have incorporated BrdU, immunohistochemistry was employed using antibodies to characterize stem cells, progenitors, neurons, astrocytes, and oligodendrocytes. Following euthanasia 5 days after BrdU incorporation, brain sections were processed for single or double label fluorescent immunohistochemistry with BrdU. Although positive labeling was seen for the neuronal (HuC/D and NeuN), astrocytic (GFAP, Vimentin) and oligodendrocytic (ASPA) antibodies throughout the goldfish brain, co-localization of these antibodies with BrdU was not observed. A number of other immunohistochemical antibodies were tried in the single label

immunohistochemistry (see Tables on pages 26, 32, and 37) but these antibodies did not label the goldfish brain and were not used for the co-localization with BrdU studies.

Figure 11. Double label with phospho-histone (H3) confirms BrdU positive cells are mitotic cells. Normal fish were killed 16 hours after continuous BrdU administration and processed for fluorescent immunohistochemistry. A. Cresyl violet stained section. Arrow denotes boxed region enlarged in B - D. B. BrdU (green arrowheads) positive cells in the molecular layer of the cerebellum. C. H3 (red arrows) labeled cell. D. DAPI nuclear label (blue). E. Merged imaged demonstrating double label for BrdU and H3 (Arrows). F. Cresyl violet stained section. Boxed region enlarged in G - J. G. BrdU (green arrowheads) positive cells in the molecular layer of the cerebellum. H. H3 (red arrows) labeled cell. I. DAPI nuclear label (blue). J. Merged imaged demonstrating double label for BrdU and H3 (Arrows).



EFFECTS OF TRANYLCTYPROMINE ON MITOTIC CELLS IN PROGENITOR ZONES

Statistical analysis of the density of BrdU positive cells demonstrated that both short and long term TCP administration significantly increased the number of BrdU positive cells within the combined brain regions examined (TCP short term, $147,200 \pm 51,020$; TCP long term: $24,390 \pm 5,298$ BrdU labeled cells; mean $\pm$ SEM) relative to control (control short term: $62,000 \pm 7,503$; control long term $7,739 \pm 1373$ BrdU labeled cells; mean $\pm$ SEM) ($p= 0.0266$; $p= 0.0114$ respectively) (Figures 12A, 14A).

To determine whether an increase in the number of BrdU positive cells was specific to a certain brain region, the telencephalon, optic tectum and cerebellum were investigated for both the short and long term administration of TCP (Figures 12B, 14B). For both of these groups and their respective controls, BrdU was administered continuously for 16 hours following the last injection and fish were immediately euthanized. In the short term treatment group, BrdU labeled cells were found to be significantly increased in the cerebellum of the TCP exposed goldfish relative to control ($p = 0.0338$; TCP: $75,480 \pm 35,800$; control: $15,270 \pm 2,691$ BrdU labeled cells; mean $\pm$ SEM). Specifically, the molecular and granular layers of the cerebellum contained significantly more BrdU positive cells relative to controls ($p = 0.0329$, TCP: $68,080 \pm 33,090$; control: $12,000 \pm 2,351$, and $p = 0.0398$, TCP: $7,220 \pm 2570$; control: $2,877 \pm 597$ BrdU labeled cells; mean $\pm$ SEM respectively) (Figures 12B, 13). Following long term administration of TCP,

BrdU labeled cells were found to be significantly increased in the optic tectum ($p = 0.0188$, TCP: $7,823 \pm 1,398$; control: $3,644 \pm 717$ BrdU labeled cells; mean $\pm$ SEM) (Figures 14B, 15A-B) and molecular layer of the cerebellum ($p = 0.0179$, TCP: $11,360 \pm 3,201$, control: $1,736 \pm 389$ BrdU labeled cells; mean $\pm$ SEM) (Figures 14B, 15C-F) in the TCP exposed goldfish compared to controls.

These results indicate that TCP increased proliferation after the last treatment and this effect was sustained for at least 16 hours. Additionally, these results indicated that after long exposure periods to TCP, a second brain region known to contain proliferation zones, the optic tectum, also contained a significantly increased density of positively labeled BrdU cells. However, neither the short (TCP: $20,230 \pm 6,068$; control: $17,680 \pm 2,538$ BrdU labeled cells; mean $\pm$ SEM) or long term (TCP: $4,505 \pm 1,431$; control: $1,903 \pm 486$ BrdU labeled cells; mean $\pm$ SEM) administration of TCP significantly increased proliferation in the telencephalon ($p > 0.05$) compared to controls (Figures 12B, 14B).

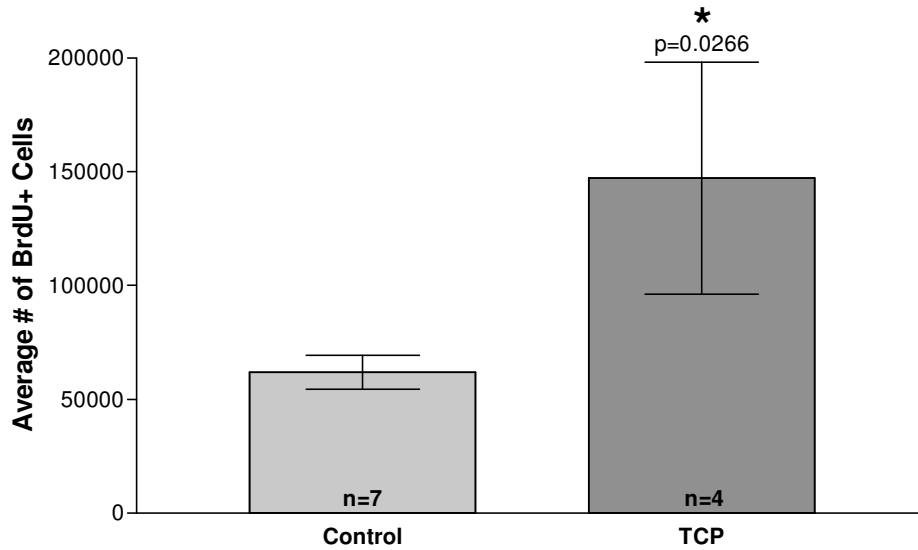
To determine whether specific brain regions maintained an increased up-regulation of progenitor cell proliferation BrdU positive cells following short or long term administration of TCP, goldfish were examined five days after BrdU exposure (Figure 16). For both of these groups and their respective controls, BrdU was administered continuously for 16 hours following the last injection and fish were euthanized five days later. The density of BrdU positive cells was not significantly different between the treatment group (TCP 5d + 5d BrdU: $27,870 \pm 6,329$; TCP 5d + 21d BrdU: $18,280 \pm 5,464$ BrdU labeled cells; mean $\pm$ SEM) and its respective control (control 5d + 5d BrdU: $22,680 \pm 6,901$; control 5d + 21d

BrdU: $13,110 \pm 1,357$ BrdU labeled cells; mean $\pm$ SEM) for the brain regions examined ($p > 0.05$).

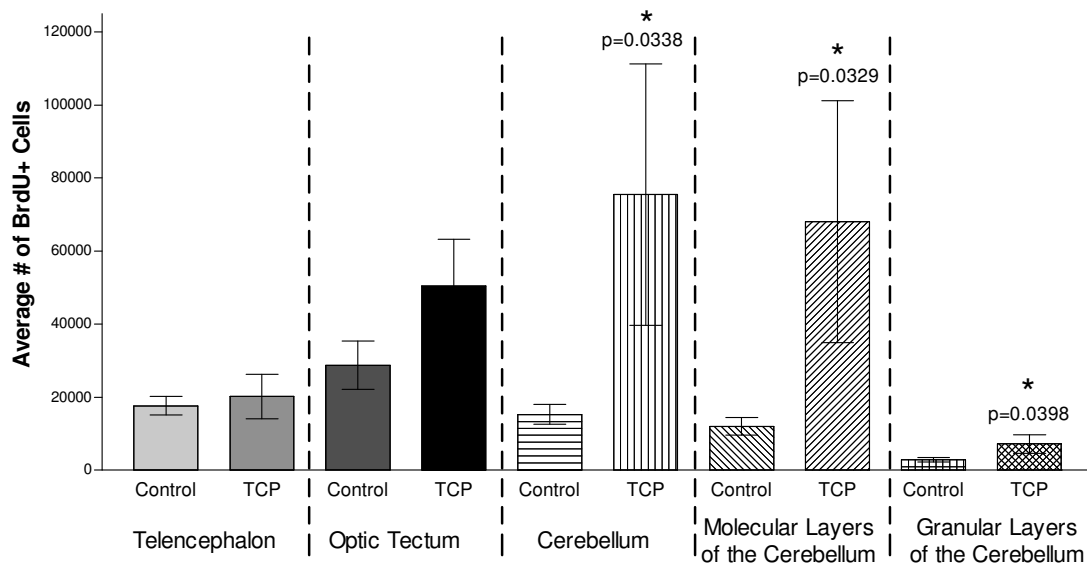
To determine whether there was an effect of treatment, comparison of short versus long term antidepressant treatment after BrdU administration was performed (Figure 17). Goldfish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)) and were killed immediately after BrdU administration. ANOVA revealed significant effects of antidepressant treatment ($p = 0.0040$). Differences between the groups were calculated by Tukey post hoc test. The short term TCP treatment had a significantly greater number of BrdU positive cells as compared to the long term control and TCP (* $p < 0.01$; ** $p < 0.05$ respectively Figure 17A). Goldfish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)) and were killed 5 days after BrdU administration. There was no significant difference among the short and long term treatment groups with a 5 day delay kill after BrdU administration ($p = 0.4198$). These data indicate that short term TCP treatment increases proliferation, as detected by the number of BrdU labeled cells compared to the long term treatment after 16 hours of BrdU administration.

Figure 12. Short term antidepressant treatment increases the number of BrdU positive labeled cells. Fish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)). The results are expressed as the mean of BrdU-positive cells in all brain regions examined (A), including the telencephalon, optic tectum, and cerebellum; or in specific regions of the brain (B). A. The average number of BrdU-positive cells in the combined brain regions was significantly increased in the TCP treated fish as compared to the vehicle control (student's t-test, * $p = 0.0266$). B. The average number of BrdU-positive cells in the cerebellum was significantly increased in the TCP treated fish as compared to the control (student's t-test, * $p = 0.0338$), where both the molecular and granular layers were significantly greater (* $p = 0.0329$ and * $p = 0.0398$ respectively. Bar = $\pm$ SEM.

A. Short term (5d) Treatment + 16hrs BrdU
 Combined brain regions: Telencephalon, Optic Tectum, and Cerebellum



B. Short term (5d) Treatment + 16hrs BrdU
 Brain regions: Telencephalon, Optic Tectum, and Cerebellum



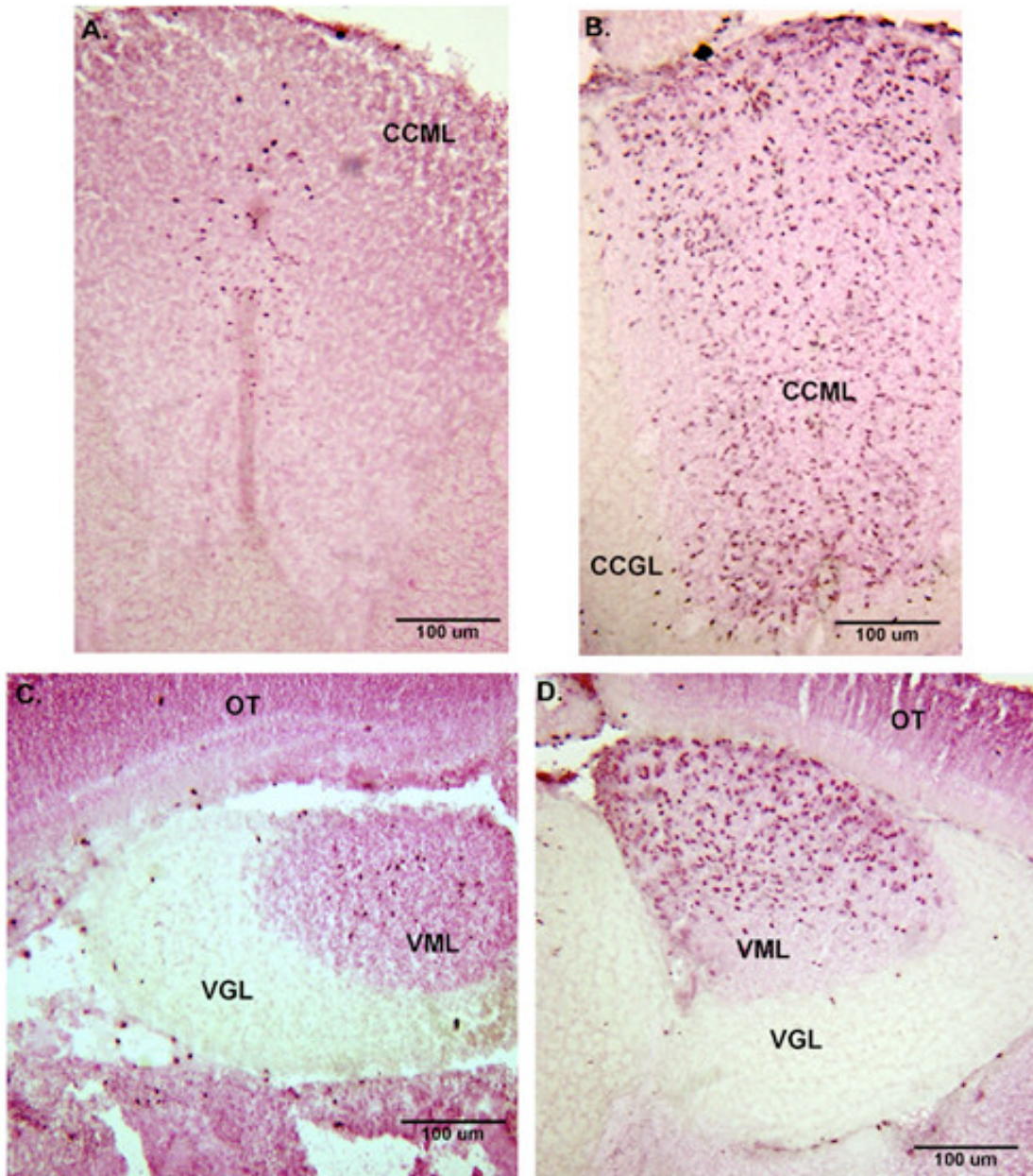
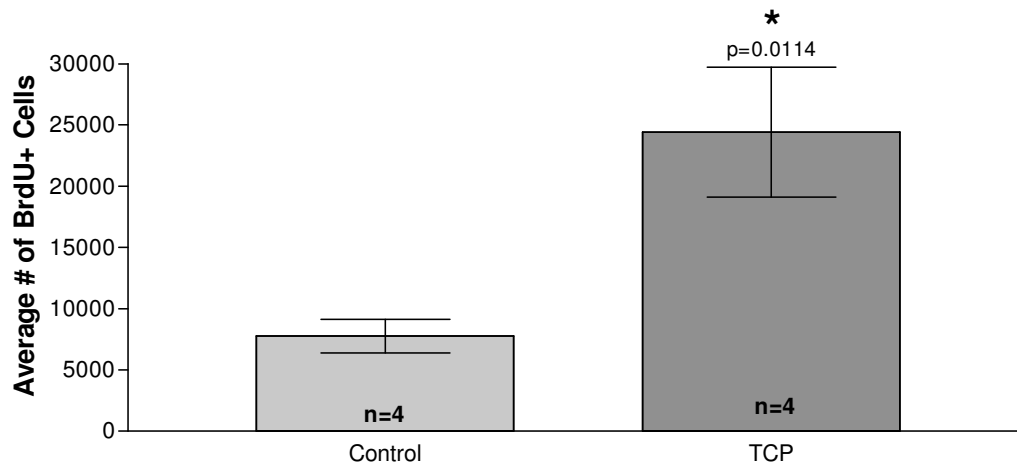


Figure 13. Short term antidepressant treatment increases the number of BrdU positive labeled cells in the cerebellum. Fish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)). A, B. BrdU-positive cells in the molecular layer of the caudal cerebellum. C., D. BrdU-positive cells in the molecular and granular layer of the valvula cerebellum. A significant increase in the number of BrdU-positive cells were found in the TCP group (B, D) compared to the Control group (A,C) in the molecular and granular layers of the cerebellum.

Figure 14. Long term antidepressant treatment increases the number of BrdU positive labeled cells. Fish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)). The results are expressed as the mean of BrdU-positive cells in all of the brain regions examined (A), including the telencephalon, optic tectum, and cerebellum; or in specific regions of the brain (B). A. The average number of BrdU-positive cells in the combined brain was significantly increased in the TCP treated fish as compared to the vehicle control (student's t-test, * $p = 0.0114$). B. The average number of BrdU-positive cells in the optic tectum and cerebellum was significantly increased in the TCP treated fish as compared to the control (student's t-test, * $p = 0.0188$ and * $p = 0.0179$ respectively). The molecular layer of the cerebellum was significantly greater (* $p = 0.123$) than controls. Bar = $\pm$ SEM.

A. Long term (5d) Treatment + 16hrs BrdU
Combined brain regions: Telencephalon, Optic Tectum, and Cerebellum



B. Long term (21d) Treatment + 16hrs BrdU
Brain Regions: Telencephalon, Optic Tectum, and Cerebellum

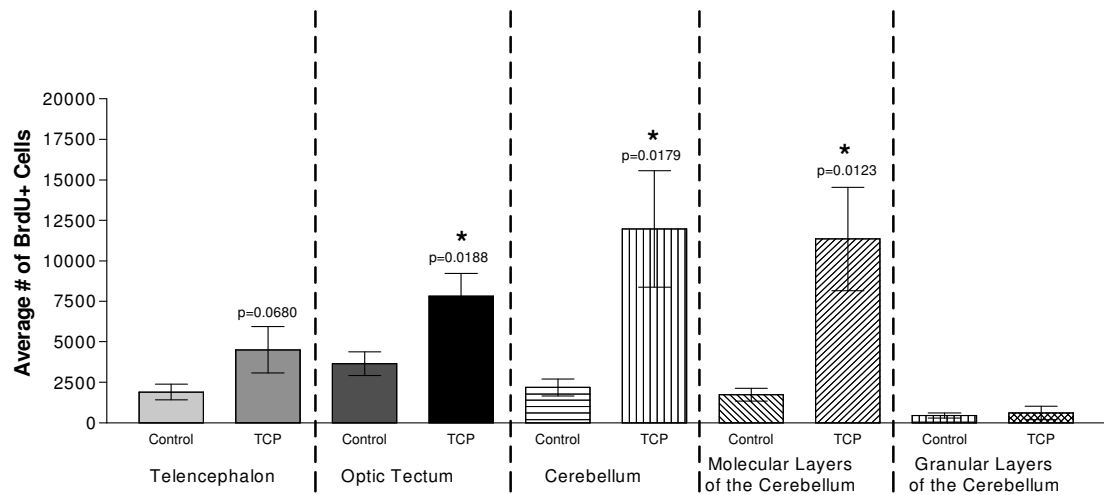


Figure 15. Long term antidepressant treatment increases the number of BrdU positive labeled cells in the optic tectum and cerebellum. Fish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)). A, B. BrdU-positive cells in torus longitudinalis (TL) of the optic tectum. C-F. BrdU-positive cells in the molecular layers of the valvula cerebellum (C, D) and caudal cerebellum (E, F). A significant increase in the number of BrdU-positive cells was found in the TCP group (B, D, F) compared to the Control group (A, C, E) in the optic tectum and molecular layers of the cerebellum. CCGL, granular layer of the corpus cerebellum; CCML, molecular layer of the corpus cerebellum; OT, optic tectum; VGL, granular layer of the valvula cerebellum; VML, molecular layer of the valvula cerebellum.

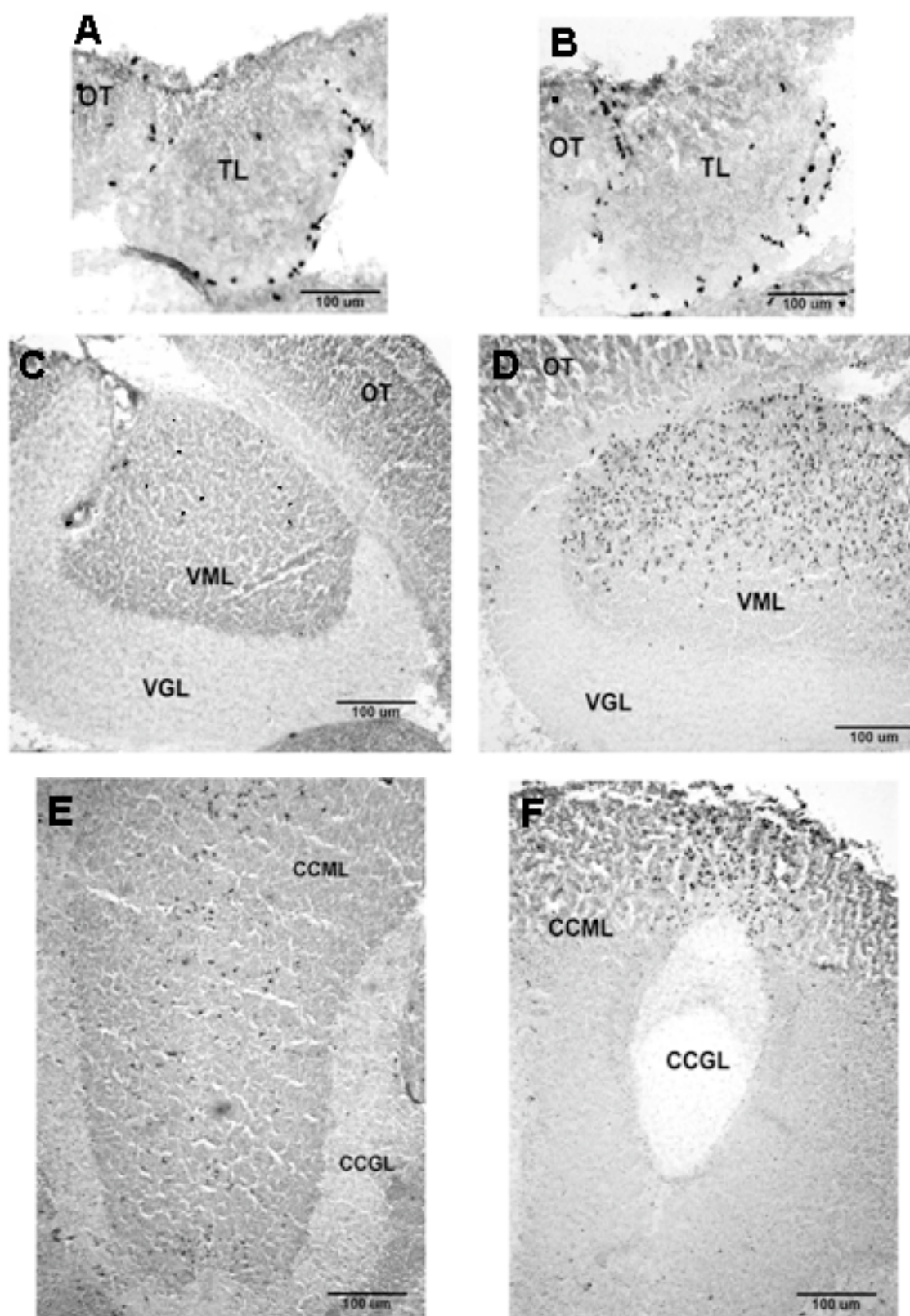
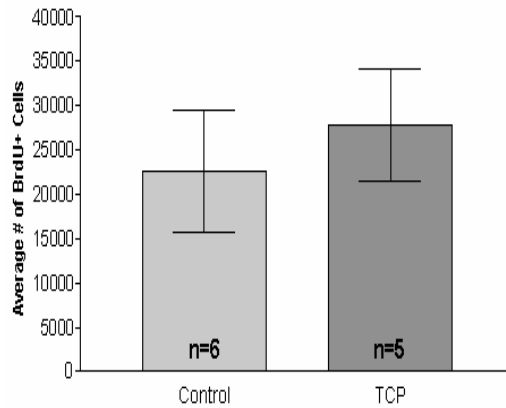
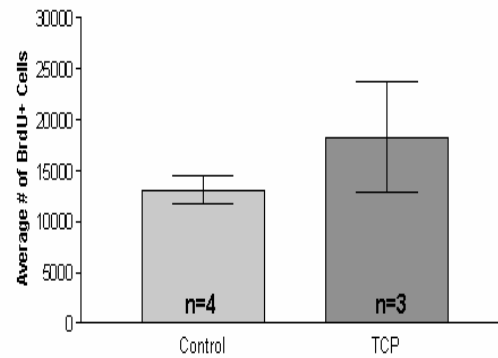


Figure 16. Short and long term antidepressant treatment with a delayed kill after BrdU administration does not increase the number of BrdU positive labeled cells. Fish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)) and were killed 5 days later. The results are expressed as the mean of BrdU-positive cells in all of the brain regions examined (A, B), including the telencephalon, optic tectum, and cerebellum; or in specific regions of the brain (C, D). The average number of BrdU-positive cells in the combined brain was not significantly different between the two groups after short term (A) or long term antidepressant treatment (B). There was no significant difference in any specific brain region examined following short term (C) or long term antidepressant treatment (D). Bar = $\pm$ SEM.

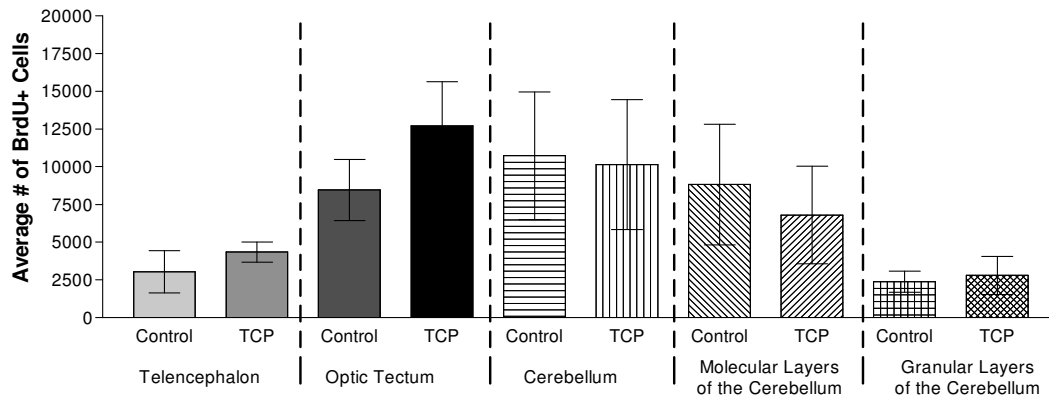
A. Short term (5d) Treatment + Killed 5d after BrdU
Combined Brain Regions: Telencephalon, Optic Tectum, and Cerebellum



B. Long term (21d) Treatment + Killed 5d after BrdU
Combined Brain Regions: Telencephalon, Optic Tectum, and Cerebellum



C. Short term (5d) Treatment + 5d after BrdU
Brain Regions: Telencephalon, Optic Tectum, and Cerebellum



D. Long term (21d) Treatment + Killed 5d after BrdU
Brain Regions: Telencephalon, Optic Tectum, and Cerebellum

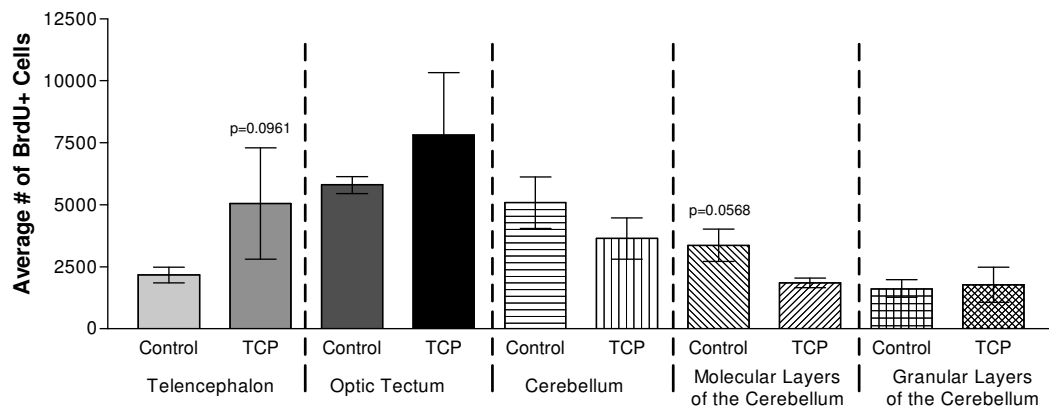
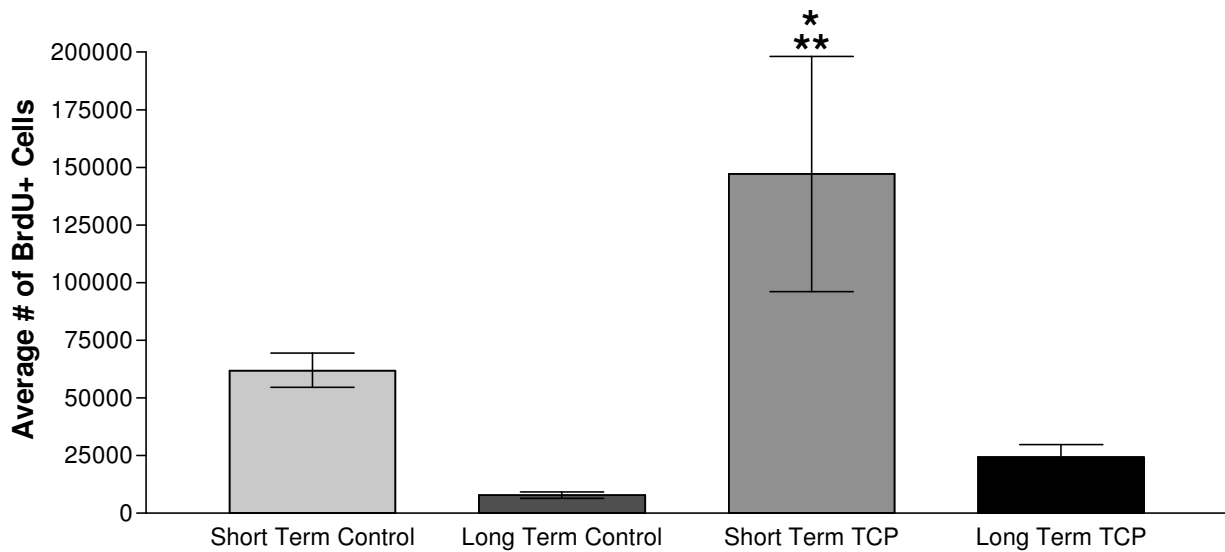
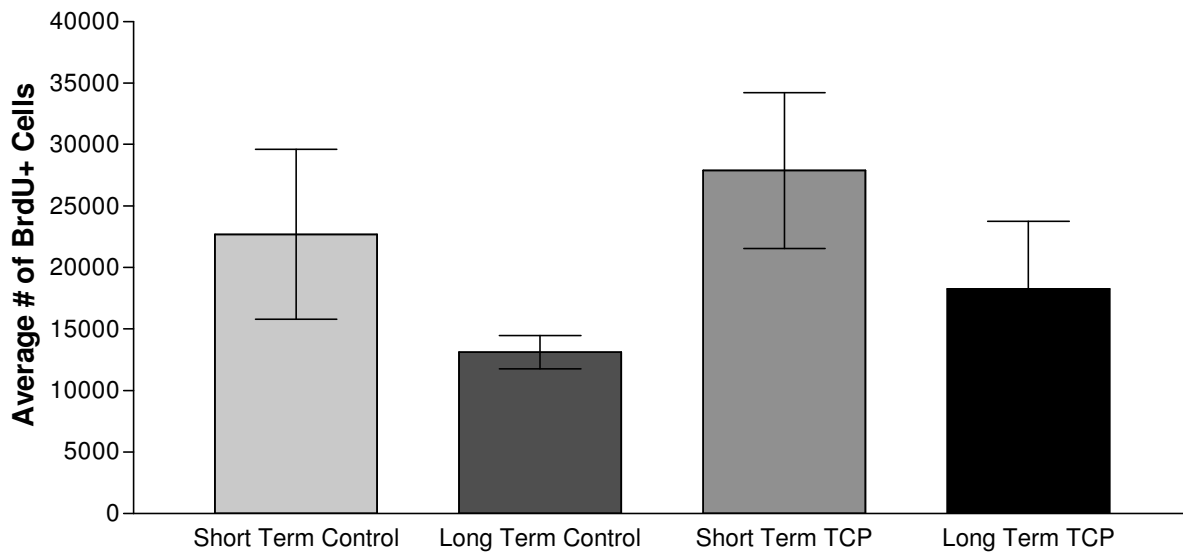


Figure 17. Comparison of short versus long term antidepressant treatment after BrdU administration. Fish received BrdU continuously for 16 hours after the last treatment (tranylcypromine (TCP) or saline (controls)) and were killed either immediately after BrdU administration or 5 days later. (A) ANOVA revealed significant effects of antidepressant treatment ($p = 0.0040$). Differences between the groups were calculated by Tukey post hoc test. The short term TCP treatment had significantly greater BrdU positive cells as compared to the long term control and TCP (* $p < 0.01$; ** $p < 0.05$ respectively). (B) ANOVA did not reveal significance among the treatment groups with a 5 day delay kill after BrdU administration ($p = 0.4198$).

**A. Short term (5d) vs. Long term (21d) Treatment + 16 hrs after BrdU
Combined brain regions**



**B. Short term (5d) vs. Long term (21d) Treatment + 5d after BrdU
Combined brain regions**



Cells migrate out of the proliferation zones into associated brain nuclei

The mobility of the mitotic cells' progeny was examined in control goldfish injected with saline control and goldfish treated for 5 consecutive days with TCP. To determine whether the BrdU labeled cells had migrated away from a proliferation zone, BrdU was administered continuously for 16 hours, and fish were either euthanized one or five days later. The 1 d time point was used as the baseline, indicating the location of BrdU labeled cells in proliferative zones. After 5 d, there was a clear spatial separation between cells in the molecular layer and cells in the granular layer as compared to the 1d (Figure 18) for both control and TCP. The pattern of movement in the cerebellum proceeds from the molecular layer to its associated granular layer. Upon qualitative examination, there was no difference in migration between TCP and control.

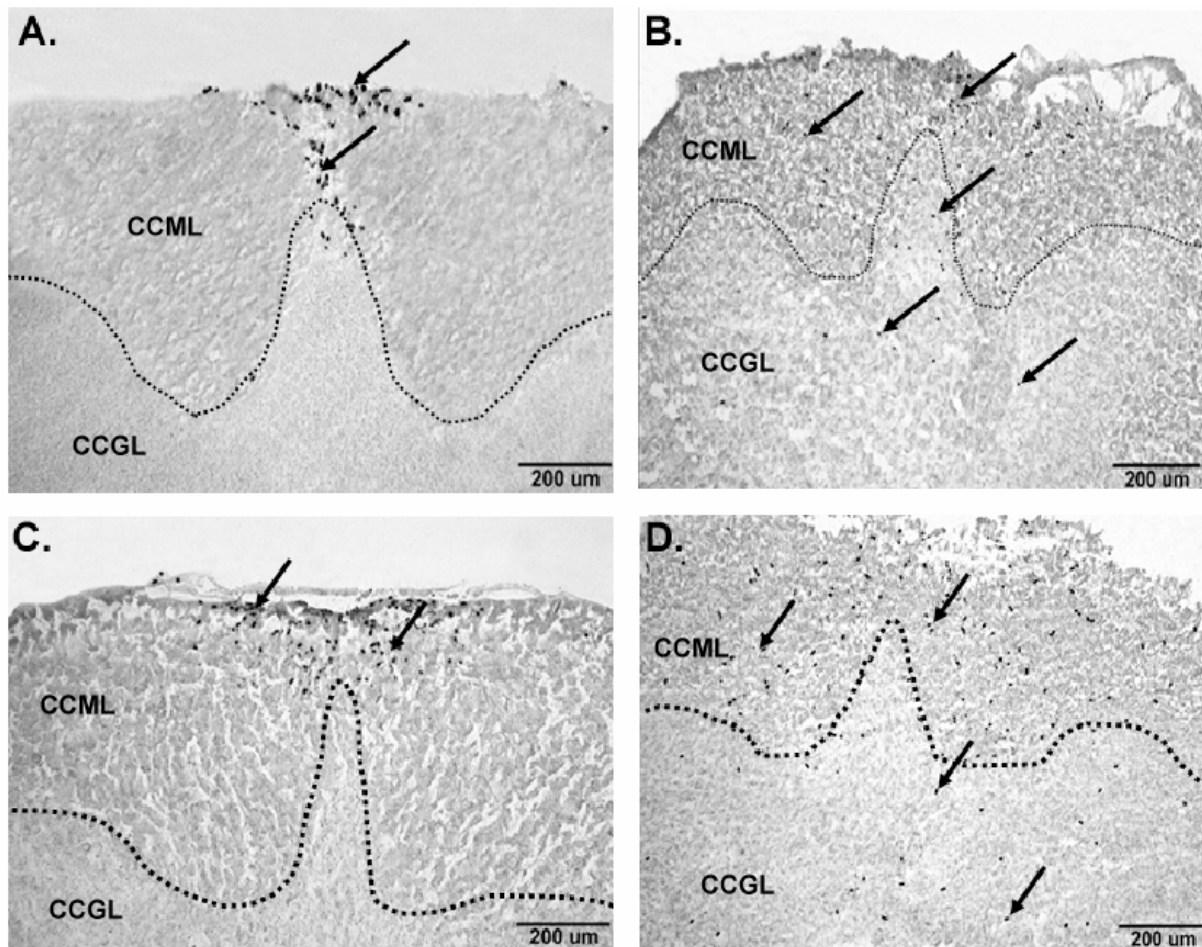


Figure 18. In both normal and tranlycypromine (TCP) treated goldfish, BrdU positive cells migrate from the molecular to the granular layer in the caudal cerebellum. A, C. BrdU positive labeled cells (arrows) in the center of the molecular layer in the caudal cerebellum at 1 day post BrdU administration. B, D. BrdU positive labeled cells (arrows) 5 days post BrdU administration. Note the presence of BrdU positive cells within the granular layer as well as being spread out in the molecular. Arrows indicate BrdU labeled cells. A, B. Normal control goldfish. C, D. Short term (5 consecutive days) TCP treated goldfish. CCGL, granular layer of the caudal cerebellum; CCML, molecular layer of the caudal cerebellum.

Apoptotic Profiles

We were interested in whether apoptotic cell death could be observed following administration of BrdU. Our initial study was with Terminal dUTP nick-end labeling (TUNEL). The TUNEL technique did not work in the goldfish brain. The BrdU positive cells processed using DAB peroxidase immunohistochemistry were examined for apoptotic nuclear profiles. Tissue was qualitatively assessed for apoptotic bodies for the control and TCP groups (Figure 19). Apoptotic cells present morphologically as shrunken nuclei that can break into smaller compact and segregated fragments with sharply delineated masses called apoptotic bodies [154]. An apoptotic body is composed of dense nuclear or cytoplasmic mass of chromatin. Apoptotic bodies were found in all of the proliferation zones. The presence of these apoptotic profiles would suggest a cell loss seen five days after BrdU in both groups. Based on qualitative examination, TCP treated goldfish had more apoptotic nuclear profiles than controls.

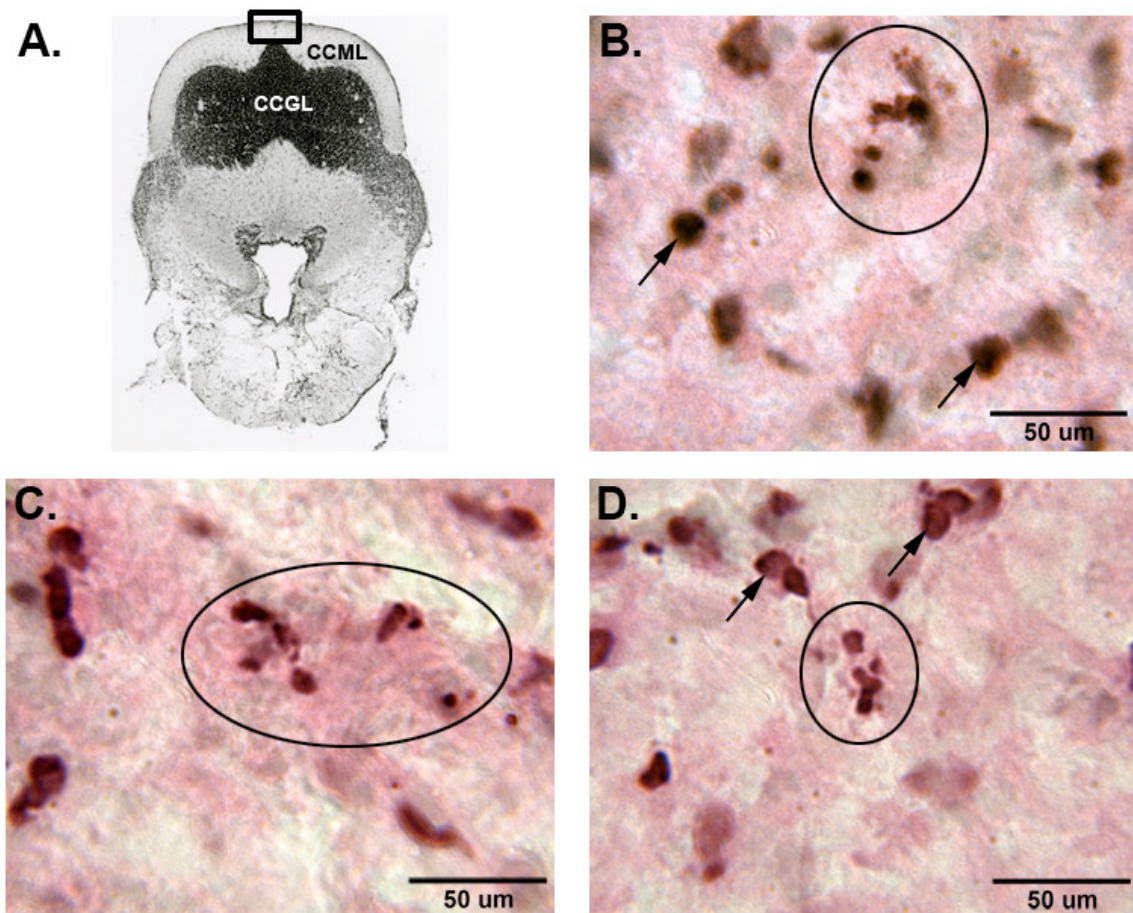


Figure 19. Apoptotic bodies detected within the caudal cerebellum. BrdU immunohistochemistry was performed on these sections and counterstained with eosin following the conclusion of TCP administration 16 hours after BrdU treatment. TCP treated goldfish presented with more apoptotic bodies than the controls. BrdU positive cells migrate from the molecular to the granular layer in the caudal cerebellum. A. Cresyl violet stained section from the molecular layer of the caudal cerebellum (CCML). Boxed region indicates area where B. – D. were photographed. B. – D. Circled areas show cells undergoing apoptosis by the presence of apoptotic bodies in the CCML of TCP treated goldfish. Arrows indicate normal BrdU positive cells. CCGL, granular layer of the caudal cerebellum

CHAPTER 4

DISCUSSION

ADULT GOLDFISH BRAIN ATLAS

An important initial step in this study was the identification and mapping of brain regions and structures through the entire goldfish brain. This map of the brain was critical for identification of proliferative zones in the normal adult goldfish. However, an atlas of the entire adult goldfish brain did not exist. We consulted several other fish atlases and published articles and developed an atlas of the goldfish brain based on cresyl violet stained sections.

Peter and Gill [127] developed a stereotaxic atlas of goldfish forebrain nuclei, which was instrumental in identifying forebrain structures. In order to identify structures within the diencephalon through the rhombencephalon, other atlases and morphological reports from various teleosts were utilized. These teleosts included zebrafish [67, 102, 136, 137, 139], brown ghost knifefish [58-60, 65, 101, 140], carp [135], three-spined fish [63], and others [126, 127, 129-131, 133-135, 141-143, 153].

The nomenclature used for this atlas was adapted from prior publications. One of the difficulties encountered with the comparison of teleost with mammalian neuroanatomy was the discrepancy in nomenclature. Teleost neuroanatomical nomenclature remained in Latin, while avian and mammalian terminology had been adapted to modern day English.

Recently, the Avian Brain Nomenclature Consortium revisited the previous terminology that defined avian brain structure and modified it to be directly linked to mammalian brain structure nomenclature [155]. Using century old terminology to define avian brain structure and evolution hindered efforts to directly link discoveries in the avian brain with those of mammals. The revision of avian brain nomenclature replaced the system developed in the 19th century by Ludwig Edinger, the father of comparative neuroanatomy [156]. Edinger's system was based on combining Darwin's theory of evolution and Aristotle's concept of a natural "scale" of organisms from lowest to highest. His system followed a chronological order of evolution where organisms of "lower" intelligence progressed into those with "higher" intelligence culminating with humans [156]. Accordingly, basic brain structures such as the spinal cord, hindbrain, midbrain, thalamus, cerebellum and cerebrum occurred in all organisms, with cerebral structures being progressively added resulting in increased encephalization [156]. Based on Edinger's system, the 'primitive' cerebral fish structures were found in all higher vertebrates, and as evolution proceeded, newer brain structures were added in amphibians, reptiles, birds and mammals.

However, in the teleost, the nomenclature remained according to Edinger, which made comparisons to the mammalian brain difficult. Additionally, the teleost has a few brain structures for which no homologous region can be identified in mammals, e.g. the valvula cerebellum and torus longitudinalis. The goldfish brain atlas presented here contains the English nomenclature as translated from the Latin and where appropriate, can be correlated to the

mammalian brain. This atlas will be extremely valuable to those researchers examining the goldfish brain.

The goldfish brain differs from other teleosts in the brain regions of the facial and vagal lobes. Compared to the zebrafish [136, 138, 139], brown ghost knifefish [140] and Japanese eel [141], the goldfish has large facial and vagal lobes [52, 129, 133, 135, 139, 157]. In a neuroanatomical comparison of the goldfish and zebrafish, Rupp et. al. [139] found the facial and vagal lobes of the goldfish correspond to an increased size in gustatory centers, relative to the zebrafish. This suggested that the goldfish has more complex gustatory centers compared to the zebrafish, which was confirmed behaviorally [139]. This suggests that the goldfish brain is evolutionary more specialized and hence, more complex than that of the zebrafish. However further comparison studies are warranted. Additionally, teleosts, including goldfish, contain two brain regions for which there is no mammalian homologue. They are torus longitudinalis and the valvula cerebellum. It has been reported that the valvula cerebellum and torus longitudinalis both receive collateral input from the lateral valvula nucleus (NLV) and functions as a link between premotor areas in the telencephalon and the brainstem [126, 142]. These comparable pathways, for which there is no mammalian homologue, may have evolved to involve different nuclei in the mammalian brain.

PROLIFERATION ZONES IN THE ADULT GOLDFISH BRAIN

Mapping and Identification of Proliferation Zones

The infraclass Teleostei contains the majority of animals commonly referred to as “fish”. The location of proliferative zones differs in mammals and teleosts due to the process of brain development. During mammalian embryonic development, the neural tube undergoes a process of evagination and inversion, or inside-in, while the teleost undergoes the process of eversion, or inside-out. [158] Due to this difference in development, many brain and periventricular structures are located on the outer portions of the brain in the teleost, which is opposite to corresponding mammalian brain structures.

Dating back to 1967, Kirsche [78] described matrix zones within the brains of the guppy, carp, and ide. These matrix zones were identified microscopically based on basic histological staining techniques and [^3H] thymidine autoradiography. Using these basic histological techniques and [^3H] thymidine autoradiography, the following matrix zones were identified: two telencephalic matrix zones (one in the dorsal and one in the ventral telencephalon), one in the preoptic region, one ventral to the habenula, another in the periventricular region of the hypothalamus, one in the caudal hindbrain, three in the optic tectum and a complex system in the cerebellum. Kirsche’s interpretation of matrix zones (progenitor zone locations) in the teleost brain was later confirmed with tritiated thymidine studies in zebrafish [159], guppy [160, 161] and goldfish [56]. Meyer [56] used tritiated thymidine to determine proliferation zones in the retina and

optic tectum in goldfish. Because these studies were focused on cell proliferation in the adult teleost brain, rather than precise anatomical descriptions, comparisons to recent results are difficult. To make matters worse, there was no consensus to nomenclature of the teleost brain nuclei. With the comprehensive analyses by several groups from the mid-1980s to mid-1990s a consensus was attainable, with some minor controversies [162, 163].

Although there have been more recent studies carefully describing proliferation zones in teleost brains (brown ghost knifefish, *Apteronotus leptorhynchus*, [59], three-spined stickleback fish, *Gasterosteus aculeatus*, [63], zebrafish, *Danio rerio* [64, 158], there have been no reports in the goldfish, *Cariassus auratus*. The creation of a comprehensive neuroanatomical atlas of the adult goldfish brain in this study made precise neuroanatomical mapping of the proliferation zones possible.

In this study, a qualitative assessment of the distribution of the BrdU labeled cells within the adult goldfish brain was performed. The proliferation zones detected in the goldfish by the distribution of BrdU labeled cells corresponded to the proliferation zones described in the stickleback fish [63] and zebrafish [64]. However, two exceptions were observed. The first exception was seen in the goldfish telencephalon where BrdU labeled cells were found along the ventro-lateral surfaces. There was an absence of BrdU cells in this region in the stickleback and zebrafish [63, 64]. This difference may be due to the methods utilized for BrdU administration. In both studies, the fish received a single pulse of BrdU while goldfish in the present study were exposed to continuous BrdU in

water for sixteen hours. One would expect to see more labeled mitotic cells during a continuous label than after a single pulse. In our preliminary studies when administration of a single pulse of BrdU was given, the density of BrdU labeled cells was extremely sparse, making assessment of proliferation zones difficult. However, by continuous administration of BrdU, larger numbers of positively labeled cells were revealed in the sparsely labeled areas by the pulse label. A second exception was seen in the hindbrain of the goldfish where the facial and vagal lobes contained a dense amount of BrdU labeled cells as compared to a few scattered cells located in these areas in the stickleback fish and zebrafish. This difference may be attributed to the fact that goldfish have more specialized and enlarged gustatory centers located in this region owing to their highly complex system of taste [139]. Goldfish have a palatal organ in the roof of the oral cavity that allows for chemosensory testing and sorting of food particles, which is topographically mapped in the vagal lobes (sensory and motor zones) [139]. Lastly, a single pulse may have missed slower proliferating cells whereas a continuous label of BrdU would have detected them. Recently, Grandel et al. [64] found two populations of proliferating cells in the zebrafish brain that were either BrdU+/ PCNA+ or BrdU-/ PCNA+. They proposed that the proliferation zones of the adult zebrafish brain contain different cell types characterized by different cell cycle lengths, indicating a stem cell pool [64].

Immunohistochemical Co-localization of Mitotic Markers

Proliferating cells were visualized by BrdU incorporation throughout the adult goldfish brain. The mitotic marker H3 was used to confirm the BrdU positive label. The distribution of H3 positive labeled cells corresponded with that of the BrdU positive cells, although the number tended to be smaller. Both single and double label immunohistochemistry using H3 and/ or BrdU demonstrated an overlap in positively stained cells within the proliferative zonal regions with occasional co-localization. These data correspond to positive immunohistochemical mitotic label co-localized with BrdU in both the stickleback and zebrafish [63, 64]. In those studies, PCNA was used in conjunction with BrdU in order to confirm proliferation zones. In contrast, PCNA failed to convincingly co-localize with BrdU in this study. Although PCNA has been used in previous studies in the carp [164, 165] and zebrafish [64, 136, 166], we were later informed that this is a difficult antibody (*personal communications with Dr. Jan Kaslin*).

Differentiation Pattern of Post Mitotic Cells

The differentiation pattern of the BrdU labeled cells was investigated to determine if these cells became neurons or glia. Many of the markers used were chosen based on their success in other teleosts. However, at the time points examined (16 hours and 5 days) post-BrdU incorporation, the cells failed to co-label with various antibodies for neurons and glia. Later end points would be

necessary to determine the fates of the BrdU labeled cells. Grandel et al [64] found that after a 46d chase, BrdU positive cells co-localized with HuC/D in regions adjacent to proliferation zones throughout the zebrafish brain. This suggested that these newborn cells are committed to a neuronal fate.

Additionally, these double labeled cells outnumbered the BrdU/ S100 β cells, which implicate a default to the neuronal rather than glial lineage. In another zebrafish study, Hinsch and Zupanc [138] found the BrdU labeled cells co-localized with HuC/D 446 to 656 days after BrdU administration. However, they did not look for co-localization with glial markers.

EFFECTS OF TRANYLCPROMINE ON MITOTIC CELLS IN PROGENITOR ZONES

This is the first study to demonstrate that short term (acute, 5 d) antidepressant administration increases cell proliferation in the brain of a teleost. Although other studies have investigated the effects of antidepressants in the teleost (mianserin, a second generation tetracyclic serotonin antagonist in zebrafish [167] and fluoxetine, a selective serotonin reuptake inhibitor, in medaka [168]), these studies aimed to determine their effects on the endocrine and reproductive systems respectively. In both studies, the primary concern was evaluation of the environmental impact that these pharmaceutical products had on these aquatic animals. TCP was used in a Parkinsonian study in the goldfish. Pollard et al. [95] found that TCP pretreatment before 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP) administration attenuated the accumulation of 1-methyl-4-phenylpyridinium ion (MPP^+), which would suggest an alleviation of Parkinsonian symptoms.

In rodent and primate studies of antidepressants (including TCP) and antidepressant treatment (e.g. electroconvulsive seizure (ECS)) have shown increased cell proliferation and neurogenesis in the hippocampus [85, 89, 91, 169-173]. In the goldfish, the dorsal lateral region of the telencephalon has been proposed to be the mammalian hippocampal homologue [174-178]. Based on the results from mammalian studies, we would have expected to see an increase in proliferation in the telencephalon. However, there was no significant change in the telencephalon of either group (Figures 12C and 14C). The goldfish may need to be subjected to a stimulus such as stress in order for the telencephalon to be responsive to the antidepressant. Additionally, the telencephalon may need to be exposed to the antidepressant for an extended period of time in order for this region to elicit a significant response. The telencephalon may still be critical in antidepressant treatment which will require a more detailed evaluation of the individual sub-regions that comprise the telencephalon.

The results of this study demonstrate a significant increase in cell proliferation within the optic tectum after the long term treatment of tranylcypromine and within the cerebellum of the goldfish in both the short and long term treatments of TCP (Figures 12C and 14C). The optic tectum has been previously characterized as a proliferative region that receives direct input from the retina [52, 55, 56, 103]. Because the optic tectum has been characterized to

contain proliferative zones, this region would be a candidate for the manipulation of its proliferation zones by antidepressants and other drugs.

The significant increase in BrdU labeled cells within the cerebellum implicates it as a target of antidepressants. There has been evidence implicating the mammalian cerebellum in a diverse spectrum of cognitive and emotional functions including spatial learning [179]; timing, sensory acquisition and attention [180, 181]; problem solving, error detection and language [182-184]; and emotions [185]. The teleost cerebellum has recently been suggested to play a role in emotional learning and spatial learning [186]. In addition, the fish cerebellum has been implicated in learning and memory processes that include motor conditioning and avoidance learning after lesioning [187]. The cerebellum in the adult teleost has been shown to be a region of pronounced cell proliferation in both the normal and injured (ablation) conditions [68, 69, 99, 101, 102, 188]. However, this is the first study to show cell proliferation in response to an antidepressant within the brain of a teleost.

Cells migrate out of the proliferation zones into associated brain nuclei

To determine whether TCP affected the migration of the mitotic cell progeny a qualitative study examined both control and TCP treated goldfish. Presently, there is no reported data on the effects of antidepressants on migration. In this study, the cerebellar region was selected for migration studies because in this region cells migrate longer distances compared to other brain regions where cells migrate only a few cell lengths away [58, 69, 101, 102]. No change in the normal

migratory path of the BrdU labeled cells was seen within the cerebellum between the control and TCP groups. In both control and TCP treated fish, there was an accumulation of positive BrdU cells within the molecular layer of the cerebellum at one day post BrdU administration. Five days post BrdU administration, labeled cells were dispersed throughout the molecular and granular layers of the caudal cerebellum for the control and TCP groups. These data indicated that the BrdU labeled cells had migrated, and that the administration of TCP did not alter the normal migration of these cells.

Apoptosis

Soutschek and Zupanc [100] were the first to demonstrate apoptosis, or programmed cell death, occurs in the brain of a teleost (brown ghost knifefish) exhibiting postnatal neurogenesis. Later, lesion studies in the cerebellum of the teleost [58] found that apoptosis was the mechanism for the removal of damaged cells, which was different from mammals [189]. It was suggested that this 'clean' type of death may explain, to some extent, the enormous regenerative capability of teleosts [58] in contrast to necrotic cell death in mammals which typically triggers an inflammatory response and leads to scar formation [190-194].

In the present study, the number of BrdU labeled cells in the pooled goldfish brain regions 16 hours or 5 days after BrdU administration to determine whether there was an effect of treatment. There was no significant difference in treatment detected 5 days post BrdU administration in the short and long term groups. However, an increase in BrdU labeled cells was detected in the short

term TCP group as compared to the long term TCP group 16 hours after BrdU administration (Figure 17). These results are opposite from the mammal, where a significant increase of BrdU labeled cells is seen after long term treatment. In Malberg's [89] study of antidepressant treatment on the rodent hippocampus, they too found an increase in BrdU cells, however this was following long term treatment. They did not examine the effects of short term antidepressant treatment. The increase in BrdU positive cells seen in the short term group 16 hours post BrdU administration may be attributed to the goldfish's pronounced proliferative ability. Additionally, stressors may be needed for a maintained increase in the number of BrdU labeled cells after long term antidepressant treatment.

Fluoxetine treatment of hippocampus-derived neural stem cells increased cell proliferation and protected against lipopolysaccharide (LPS)-induced apoptosis [195]. LPS is an inflammatory component used as a stress model for inducing caspase-dependent apoptosis [195]. Chiou et. al. [195] determined that fluoxetine stimulated cellular FLICE-inhibitory protein (c-FLIP) expression in the hippocampal neural stem cells, which inhibited apoptosis. Kubera et al [196] demonstrated that antidepressant drugs can inhibit the release of interleukins 1 and 6, and tumor necrosis factor alpha (TNF- α) induced by LPS. Chronic administration of lithium in rat cerebellar [197, 198], cerebral cortical [197, 199, 200] and hippocampal neurons [197] was protective against glutamate induced cell death. These data support the notion that antidepressants increase proliferation.

The mechanism of action for TCP was recently established in mammalian embryonal carcinoma cells. Briefly, TCP inhibits lysine specific demethylase 1 (LSD1), which leaves Histone 3 proteins methylated, thereby activating the pluripotential stem cell marker *Oct-4* and other stem cell-specific transcription factors [98]. Modulation of histones are hypothesized to signal changes in chromatin structure, which can result in gene expression changes and regulatory transcription complex recruitments [201]. By activating the pluripotent stem cell marker *Oct-4*, self-renewal can occur [202, 203]. This mechanism supports our finding of increased BrdU labeled cells in the goldfish brain after short term TCP administration. However, little is known about the expression of *Oct-4* in the goldfish brain, what effects antidepressants have on this gene and how regulation and/ or expression of this gene may vary between mammals and teleosts. Understanding the role that stem cell transcription factors, including *Oct-4*, play during antidepressant treatment will ultimately provide insight into the emerging link between antidepressant treatment and neurogenesis.

CHAPTER 5

SUMMARY AND CONCLUSIONS

As an initial step in this study an atlas of the adult goldfish brain was created for the purpose of identifying brain regions and structures in this teleost. The terminology used in the creation of this atlas incorporated terminology commonly used in atlases and articles of teleost and mammalian brains [58-60, 63, 65, 67, 101, 102, 126, 127, 129-131, 133-137, 139-143, 153]. Thus, this atlas will be a valuable reference for comparisons to other teleost and mammalian brains.

Several proliferation zones were identified throughout the goldfish brain using BrdU and H3 immunohistochemistry and mapping these zones using the adult goldfish brain atlas. The proliferation zones identified within the goldfish brain included the telencephalon, diencephalon, optic tectal lobes, cerebellum, facial and vagal lobes, and were in agreement with proliferation zones reported in other teleosts. The proliferation zones located within the hindbrain, which contains the facial and vagal lobes, was one region that differed from other teleosts [139]. It would be of interest to investigate this region in the goldfish to determine alterations that occur in the cellular proliferation in the facial and vagal lobes in response to their extraordinary chemosensory ability [139].

Based on individual analyses this study is the first to demonstrate that short (Group 1, acute, 5 d injection) and long (Group 3, chronic, 21 d injection) term antidepressant administration increased cell proliferation in the goldfish brain and

that this effect was sustained for at least 16 hours (Figure 14A, 16A). TCP caused a significant increase in cell proliferation in the optic tectum and the cerebellum as detected by BrdU immunohistochemistry. The teleost cerebellum has been implicated in learning and memory processes that include motor conditioning and avoidance learning after lesioning [187], and has recently been suggested to play a role in emotional learning and spatial learning [186]. The significant increase in BrdU labeled cells within the cerebellum implicates it as a target of antidepressants.

Comparison of the treatment paradigms determined that short term TCP treatment followed by 16 hour BrdU administration caused significantly more proliferation than long term TCP treatment followed by 16 hour BrdU administration (Figure 17A). The increase in BrdU positive cells seen in the short term group 16 hours post BrdU administration may be attributed to the goldfish's pronounced proliferative ability. Additionally, the significant decrease observed in the long term TCP treatment followed by 16 hour BrdU administration may be attributed to apoptosis, a process coupled with neurogenesis [108]. Apoptotic nuclear profiles in the BrdU labeled cells were seen in all the proliferation zones (Figure 19) in both control and TCP groups. TCP's mechanism of action was recently reported [92, 192] to involve the stem cell transcription factor Oct-4, and it would be of interest to investigate the role of other stem cell and proliferative factors that may be involved or affected by TCP. Because the goldfish differed from the mammal following long term treatment of TCP, a stressor applied prior to antidepressant treatment may produce results similar to the mammal.

Currently various forms of aquatic life are used to assess the environmental influences of antidepressants on reproduction and health [167, 168]. The TCP data in this study suggests that the goldfish would be an excellent model for investigating the mechanisms involved in antidepressant effects on the brain.

CHAPTER 6

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APPENDIX I

Abbreviations for Goldfish Brain Atlas

AC:	Anterior Commissure
ACT:	Anterior Cerebellar Tract
AP:	Area Postrema
APN:	Anterior Periventricular Nucleus
ATN:	Anterior Tuberal Nucleus (Nucleus Tuberalis Anterior)
BC:	Brachial Conjectivum
CC:	Cerebellar Crest (Crista Cerebelli)
CCGL:	Granular Layer of Cerebellar Corpus (Corpus Cerebelli, Stratum Granulosum)
CCML:	Molecular Layer of Cerebellar Corpus (Corpus Cerebelli, Stratum Moleculare)
CP:	Central Posterior Thalamic Nucleus (Nucleus Centralis Posterior Thalami)
Dc:	Central Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Centralis)
Dd:	Dorsal Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Dorsalis)
DF:	Nucleus Diffusus of the Inferior Lobe
DH:	Dorsal Horn
DiV:	Diencephalic Ventricle

Dm:	Medial Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Medialis)
DMNX:	Dorsal Motor Nucleus of the Vagus
Dp:	Posterior Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona Posterior)
DTN:	Dorsal Tegmental Nucleus (Nucleus Tegmentalis Dorsalis)
Dv:	Ventral Zone of Dorsal Telencephalic Area (Area Dorsalis Telencephali, Zona ventralis)
E:	Entopeduncular Nucleus (Nucleus Entopeduncularis)
FR:	Habenulointerpeduncular Tract (Fasciculus Retroflexus = Tractus Habenulointerpeduncularis)
FZ:	Fiber Zone
GE:	Granular Eminence (Ementia Granularis)
GN:	Glomerulosis Nucleus
H:	Habenular Nucleus (Nucleus Habenularis)
Hd:	Dorsal Periventricular Hypothalamus (Nucleus Periventricularis Hypothalami, Zona Dorsalis)
Hc:	Caudal Periventricular Hypothalamus
HC:	Horizontal Commissure
Hv:	Ventral Zone of Periventricular Hypothalamus (Nucleus Periventricularis Hypothalami, Zona Ventralis)
IC:	Internal Cellular Layer of Olfactory Bulb (Stratum Cellulare Internum Bulbi Olfactorii)

IMRF:	Intermediate Reticular Formation (Formatio Reticularis, Pars Intermedia)
LCAG:	Granular Layer of Caudal Lobe of Cerebellum (Valvula Cerebelli, Pars Medialis, Stratum Granulosum)
LH:	Lateral Hypothalamic Nucleus
LL:	Lateral Longitudinal Fascicle = Lateral Lemniscus (Fasciculus Longitudinalis Lateralis)
LMVC:	Molecular Layer of the Lateral Part of the Valvula Cerebelli (Lobus Caudalis Cerebelli, Stratum Moleculare)
LRN:	Lateral Recess Nucleus (Nucleus of the Lateral Recess)
LFB:	Lateral Forebrain Bundle (Fasciculus Lateralis Telencephali)
LOT:	Lateral Olfactory Tract (Tractus Olfactorius Lateralis)
LPG:	Lateral Preglomerular Nucleus (Nucleus Praeglomerular Lateralis)
LVII:	Facial Lobe (Lobus Facialis)
LX:	Vagal Lobe (Lobus Vagus)
MB:	Mammillary Body (Corpus Mamillare)
MFB:	Medial Forebrain Bundle (Fasciculus Medialis Telencephali)
MLF:	Medial Longitudinal Fascicle (Fasciculus Longitudinalis Medialis)
MON:	Medial Octavolateralis Nucleus (Nucleus Octavolateralis Medialis)
MOT:	Medial Olfactory Tract
MPN:	Medial Pretoral Nucleus
MZ:	Motor Zone
NDLI:	Inferior Lobe of the Diffusus Nucleus

NDM:	Dorsomedial Nucleus of the Thalamus
NLT:	Lateral Tuberal Nucleus (Nucleus Lateralis Tuberis)
NMLF:	Nucleus of the Medial Longitudinal Fascicle (Nucleus Fasciculus Longitudinalis Medialis)
NR:	Nucleus Rotundus
OA:	Olfactory Afferent Fibers
OC:	Optic Chiasm (Chiasma Opticum)
ON:	Optic Nerve (Nervus Opticus)
OTe:	Optic Tectum (Tecti Optici)
PC:	Posterior Commissure
PG:	Preglomerular Complex
PGZ:	Periventricular Gray Zone of Optic Tectum (Stratum Periventriculare Tecti Optici)
PPa:	Parvocellular Preoptic Nucleus, Anterior Part (Nucleus Praeopticus Parvocellularis, Pars Anterior)
PPd:	Periventricular Pretectal Nucleus, Dorsal Part (Nucleus Praetectalis Periventricularis, Pars Dorsalis)
PPN:	Posterior Periventricular Nucleus
RV:	Rhombencephalic Ventricle (Ventricularis Rhombencephali)
SZ:	Sensory Zone
T:	Tangential Nucleus (Nucleus Tangentialis)
TeV:	Tectal Ventricle (Ventricularis Mesencephali)
TGN:	Tertiary Gustatory Nucleus

TL:	Torus Longitudinal
TLa:	Torus Lateralis
TP:	Posterior Tuberal Nucleus
TPp:	Periventricular Nucleus of Posterior Tuberculum (Nucleus Periventricularis Tuberculi Posterioris)
TS:	Torus Semicircularis
V:	Ventricle
Vcm:	Medial Lobe of Valvula of Cerebellum
Vd:	Dorsal Zone of Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Dorsalis)
VDi:	Intermediate Subnucleus of Vd
VH:	Ventral Horn
VII:	Sensory Root of the Facial nerve
VIII:	Octaval Nerve (Nervus Octavus)
VI:	Lateral Nucleus of the Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Lateralis)
VLG:	Granular Layer of the Lateral Part of the Valvula Cerebelli (Valvula Cerebelli, Pars Lateralis, Stratum Granulosum)
VLM:	Molecular Layer of the Lateral Part of the Valvula Cerebelli (Valvula Cerebelli, Pars Medialis, Stratum Moleculare)
Vm:	Ventromedial Thalamic Nucleus (Nucleus Ventromedialis Thalami)
VMG:	Granular Layer of the Medial Part of the Valvula Cerebelli (Valvula Cerebelli, Pars Medialis, Stratum Granulosum)

VMM:	Molecular Layer of the Medial Part of the Valvula Cerebelli (Valvula Cerebelli, Pars Medialis, Stratum Moleculare)
Vp:	Postcommissural Nucleus of Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Postcommissuralis)
Vs:	Sensory Root of the Trigeminal Nerve (Radix Motoria Nervi Trigemini)
Vv:	Ventral Zone of Ventral Telencephalic Area (Area Ventralis Telencephali, Nucleus Ventralis)
X:	Vagal Nerve (Nervus Vagus)
Xm:	Motor Root of the Vagal Nerve
Xs:	Sensory Root of the Vagal Nerve

APPENDIX II

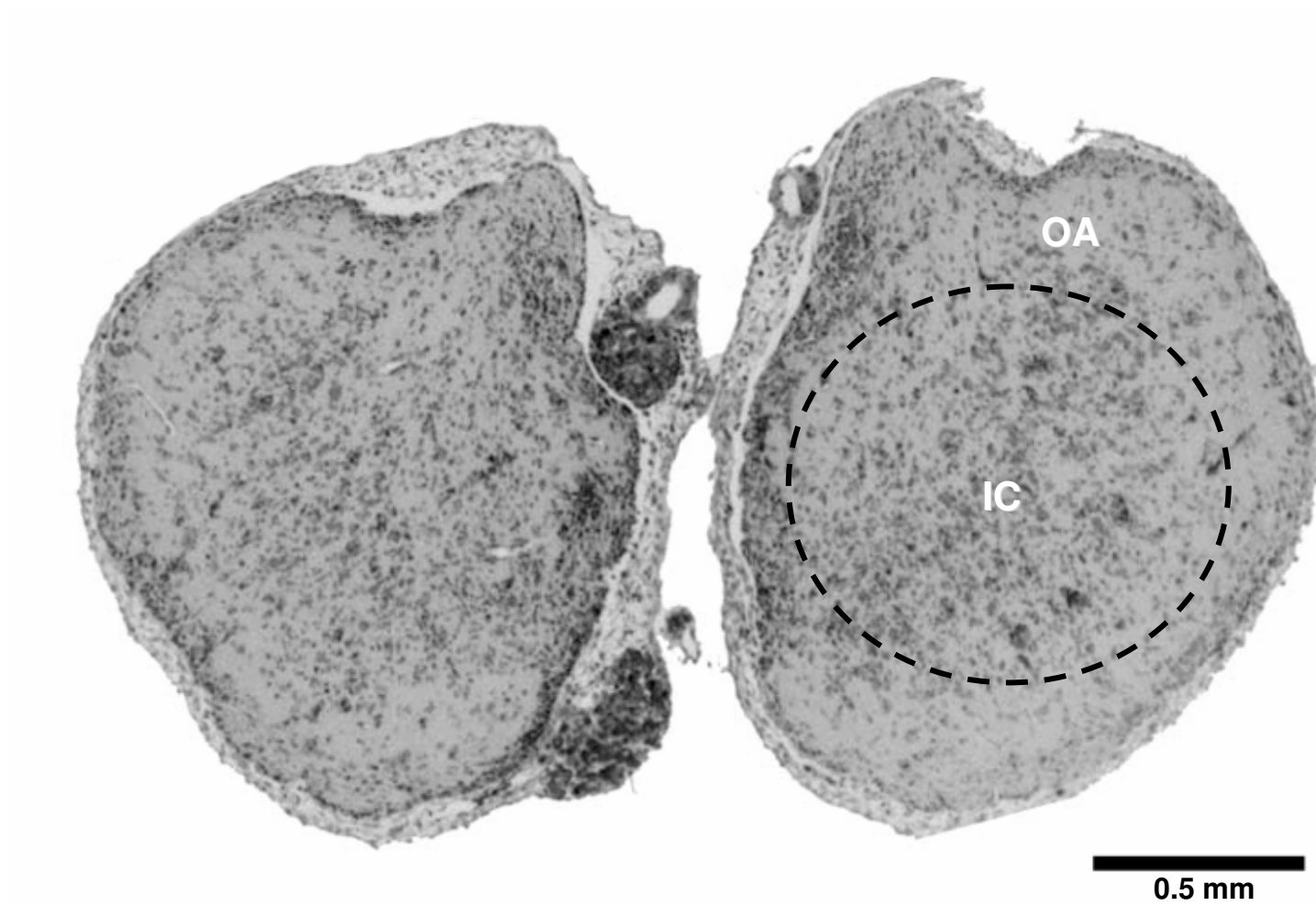
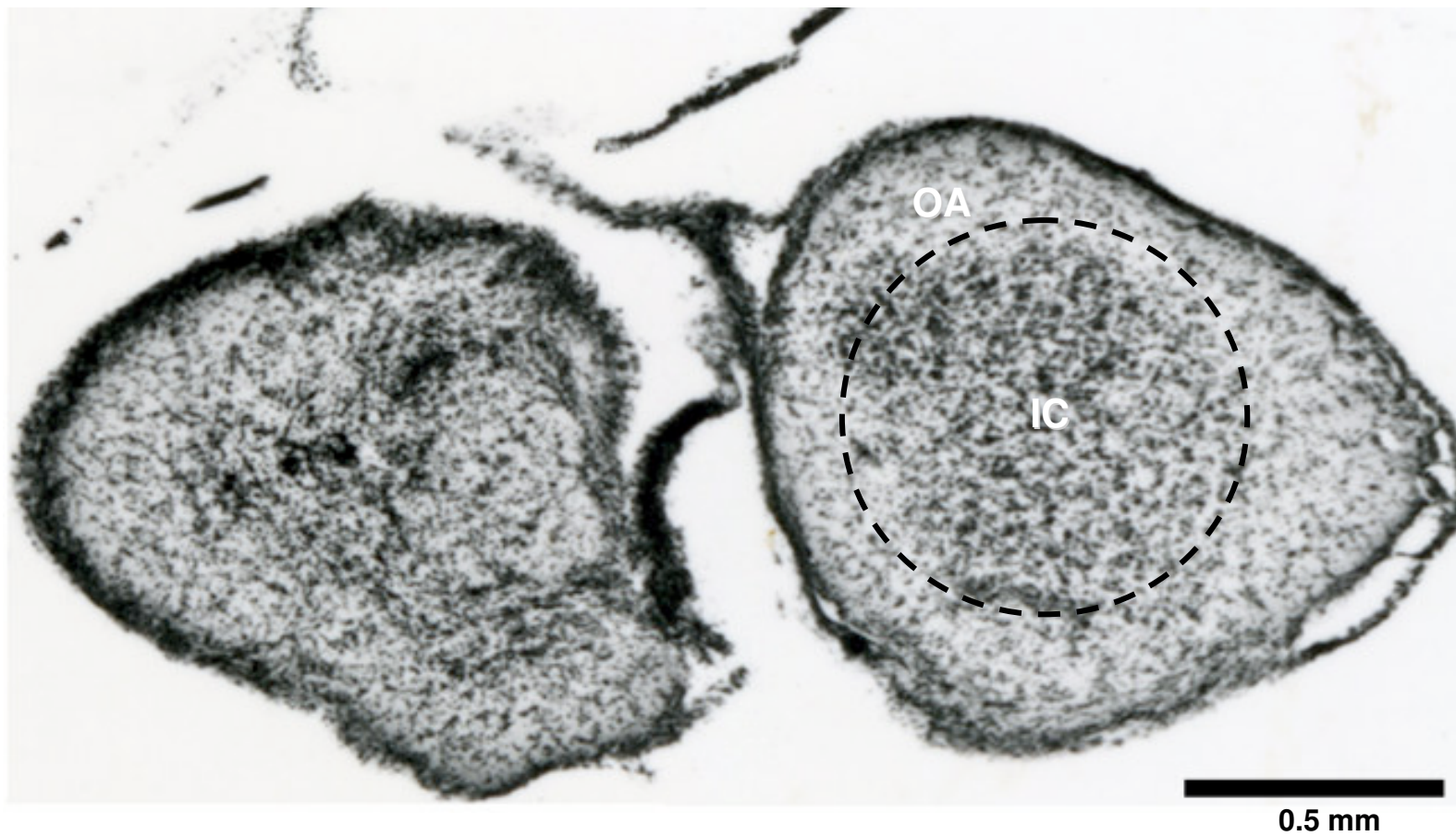


Plate 1



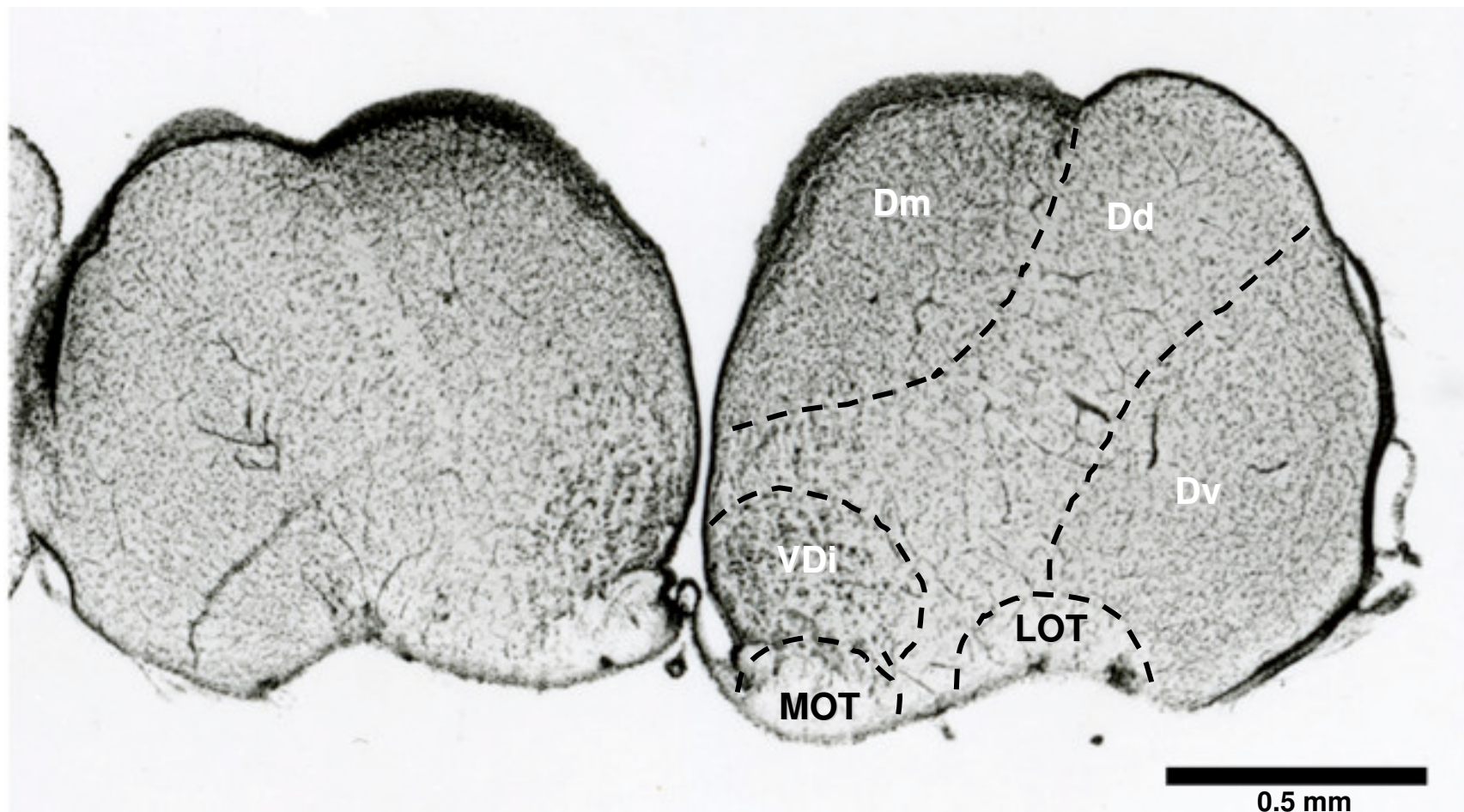
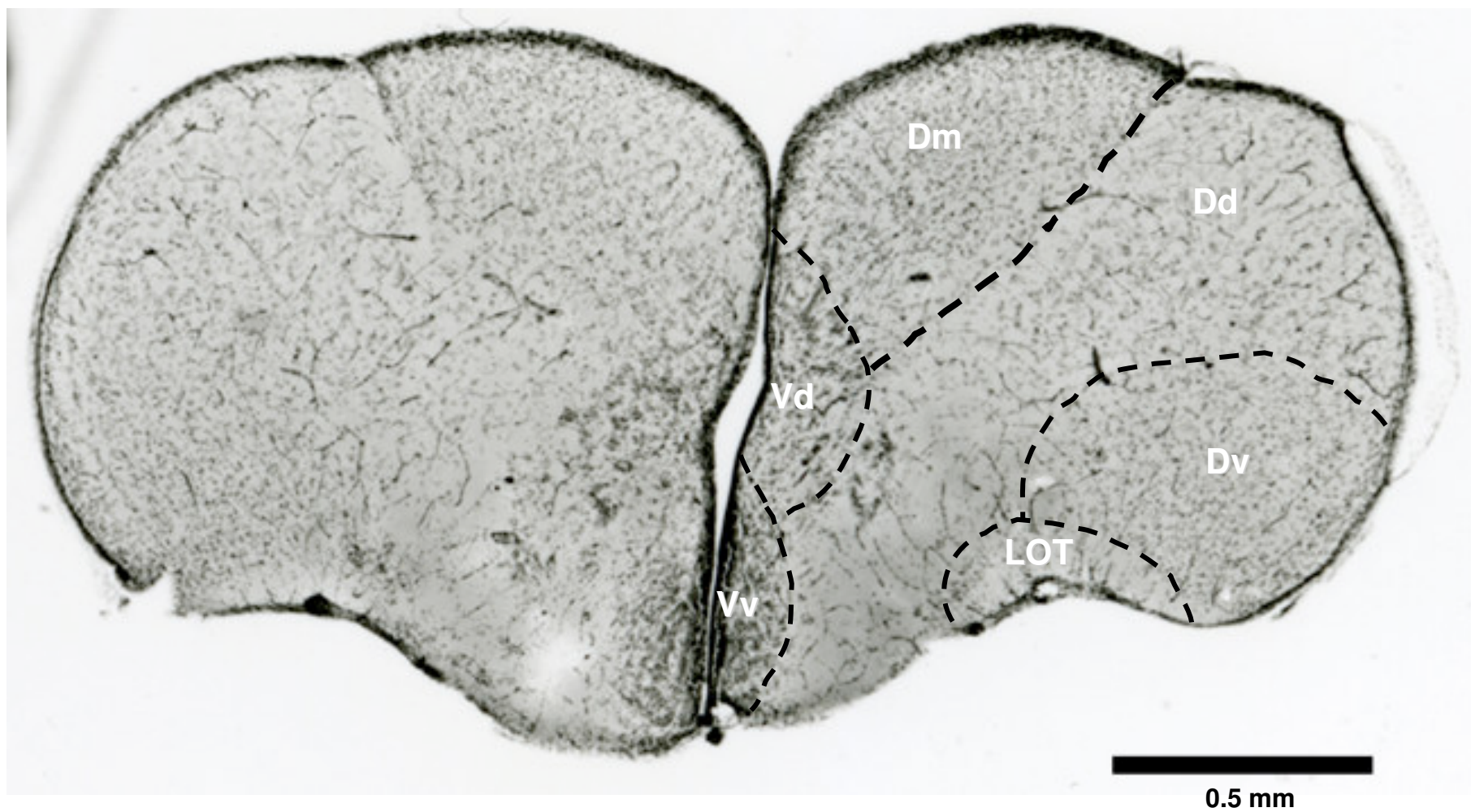


Plate 3



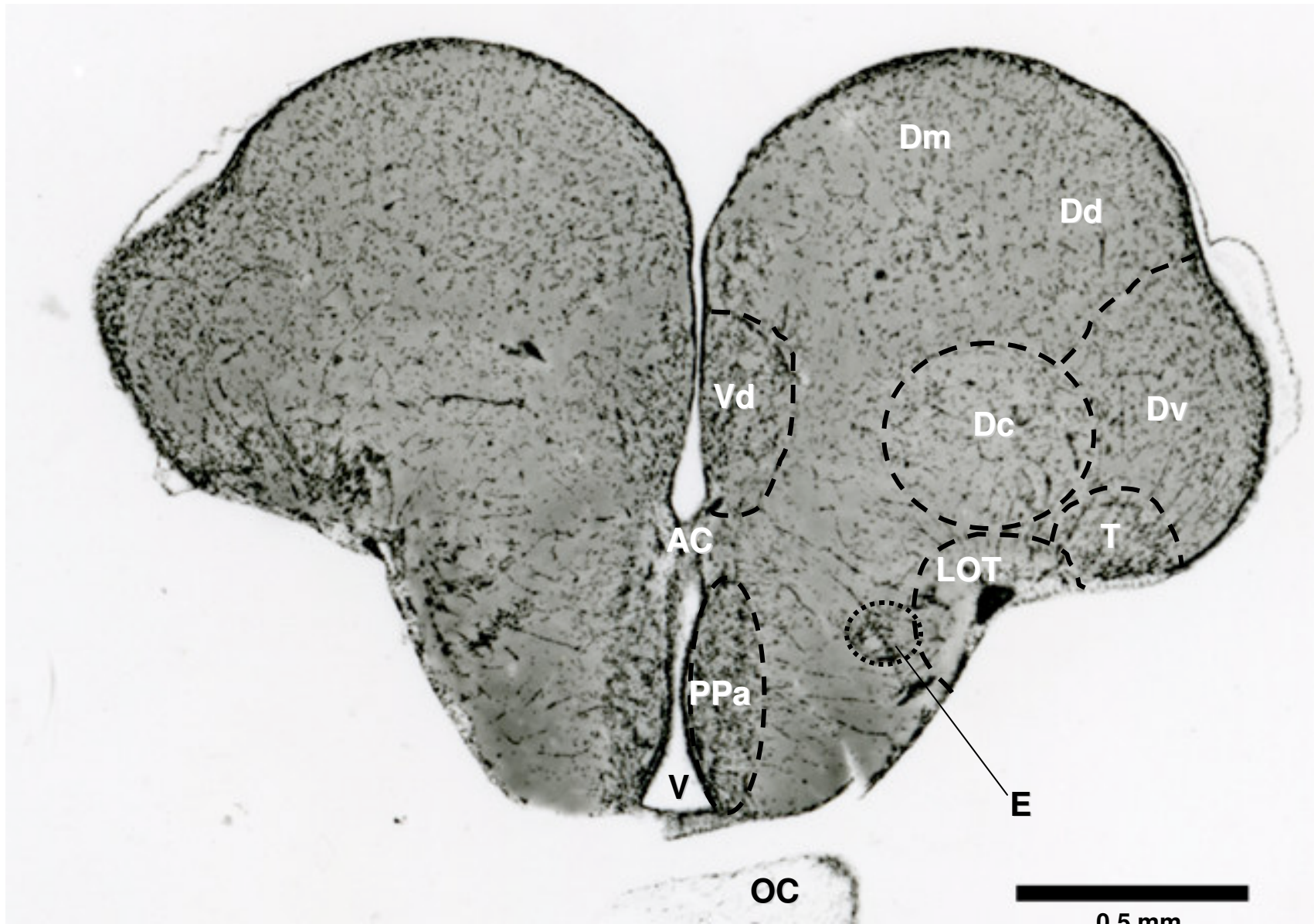
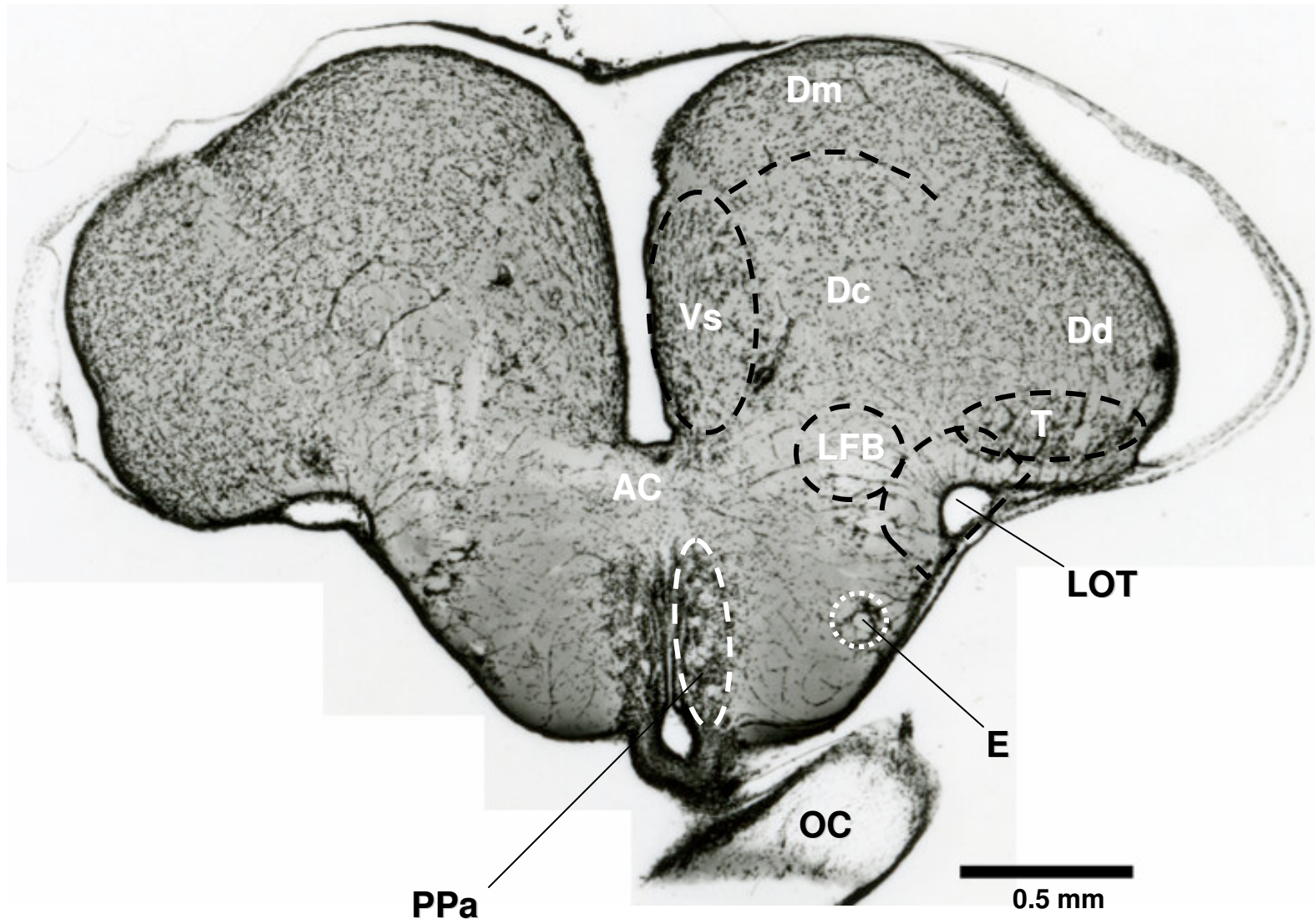


Plate 5



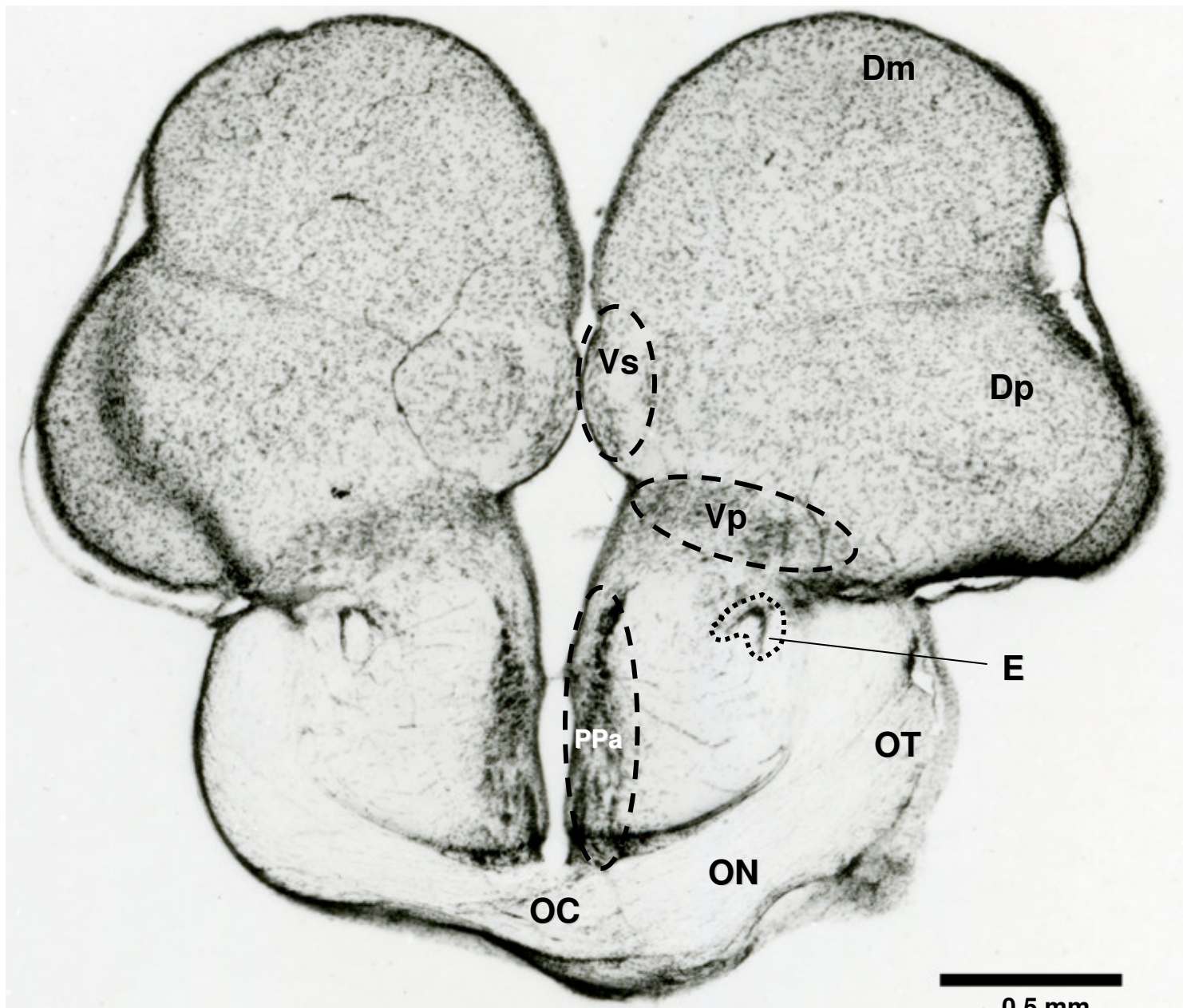


Plate 7

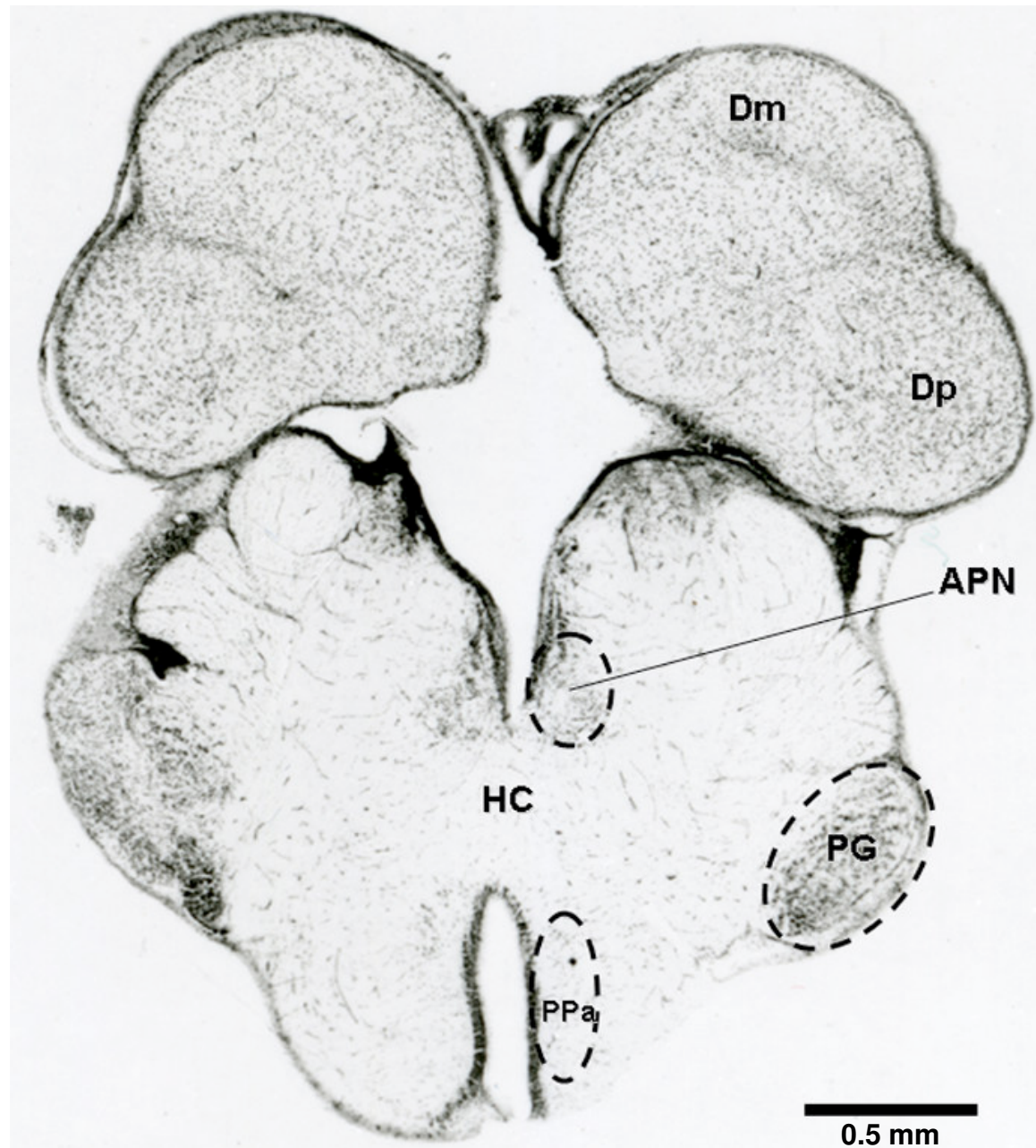


Plate 8

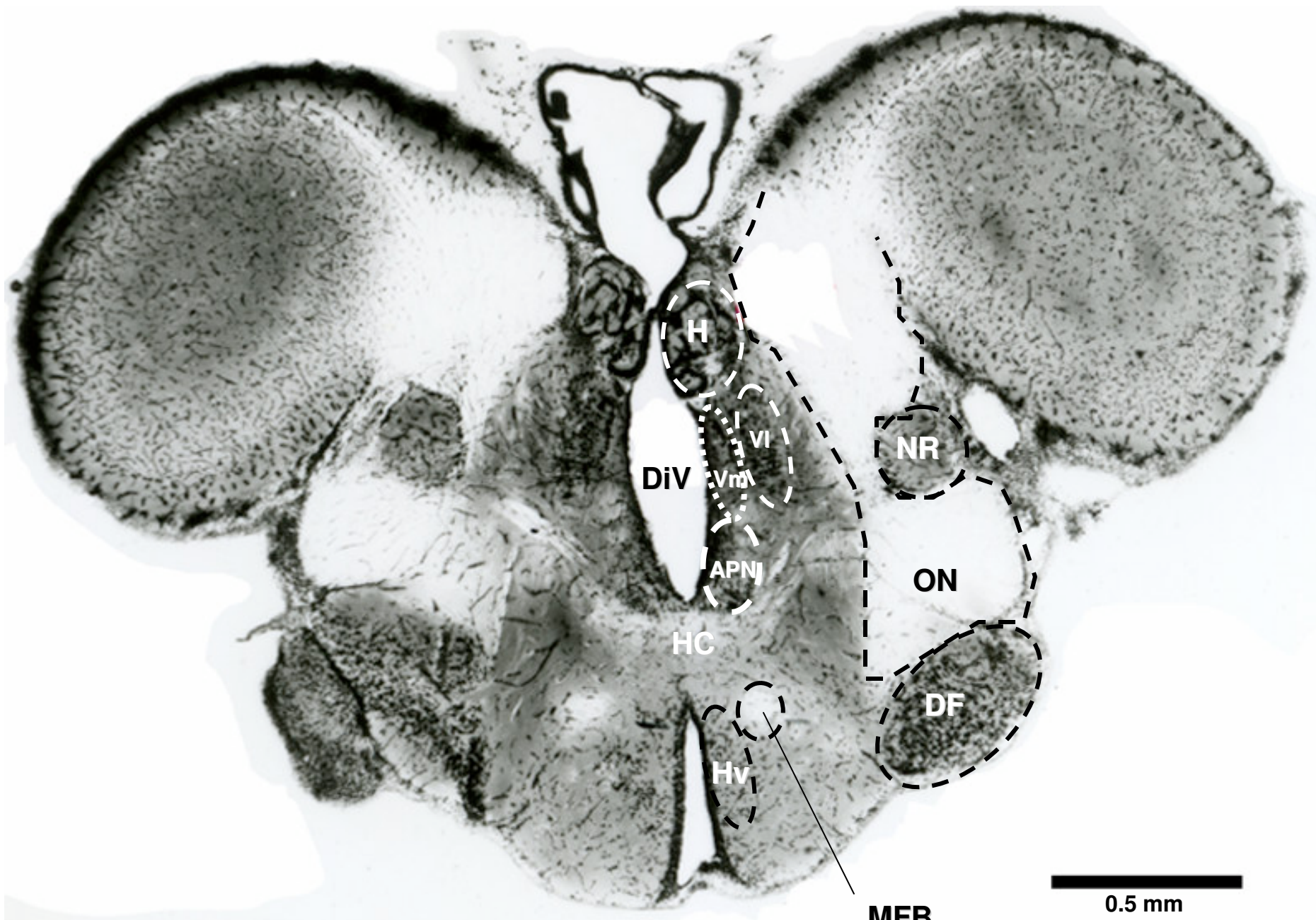


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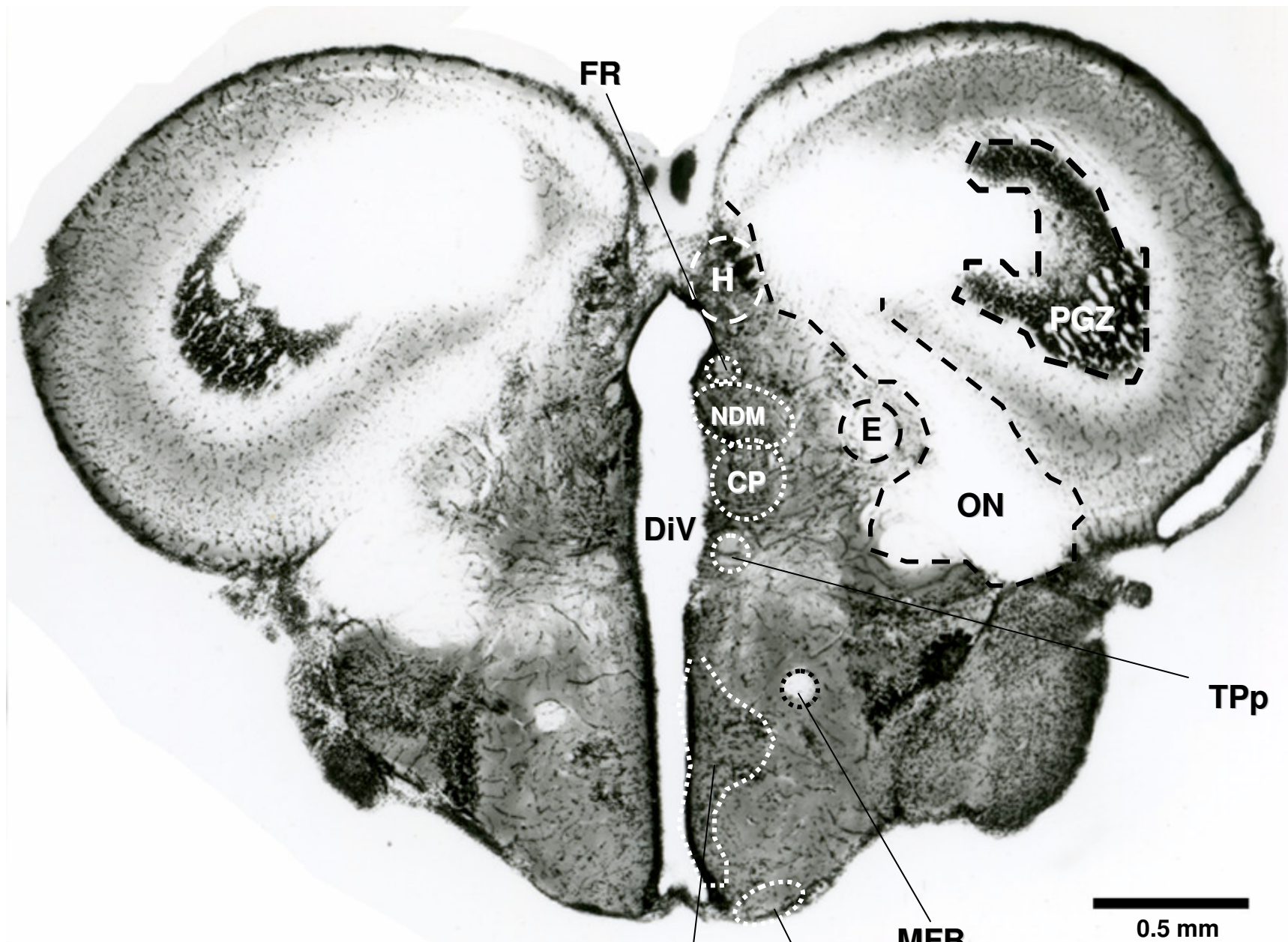


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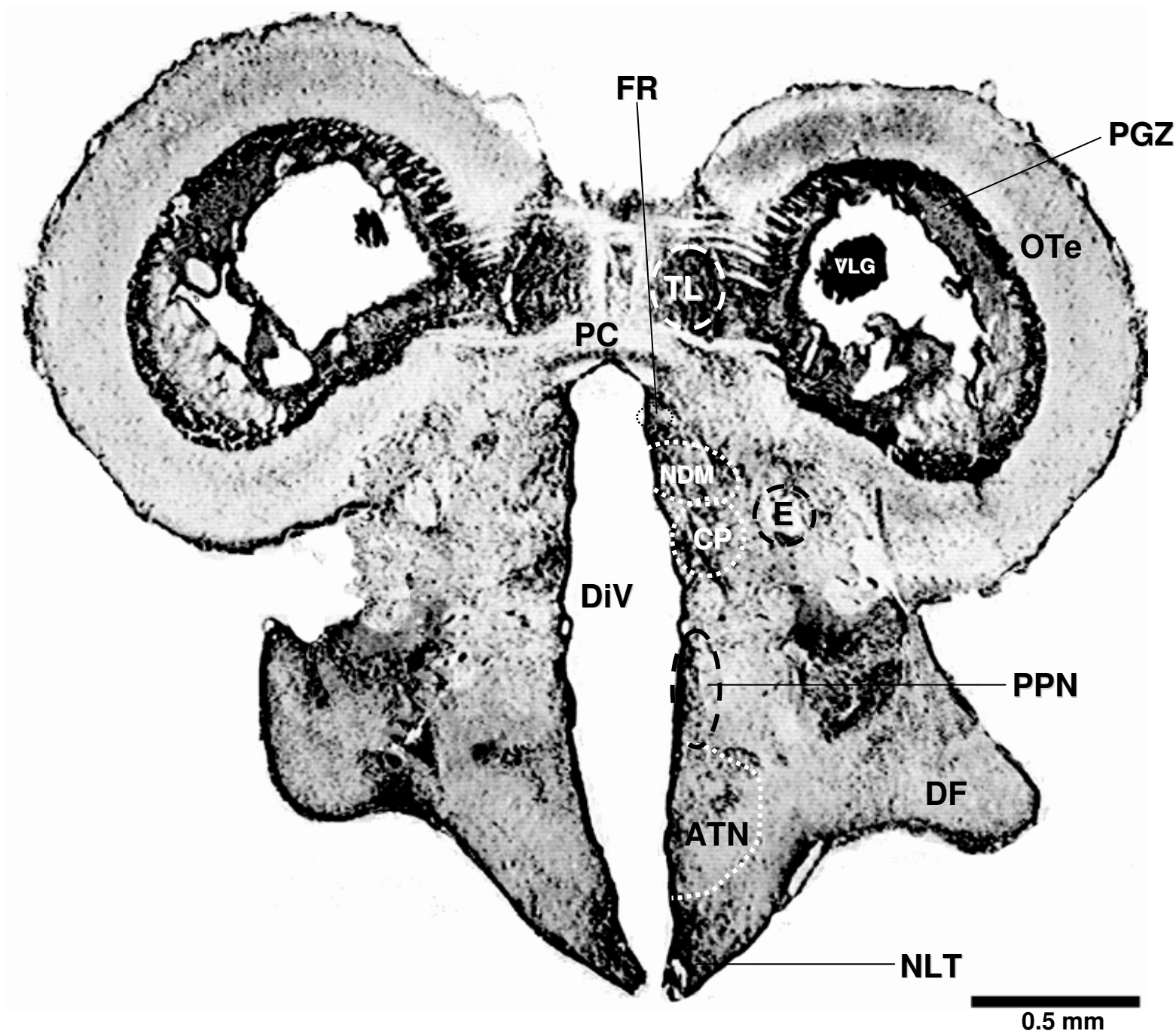


Plate 11

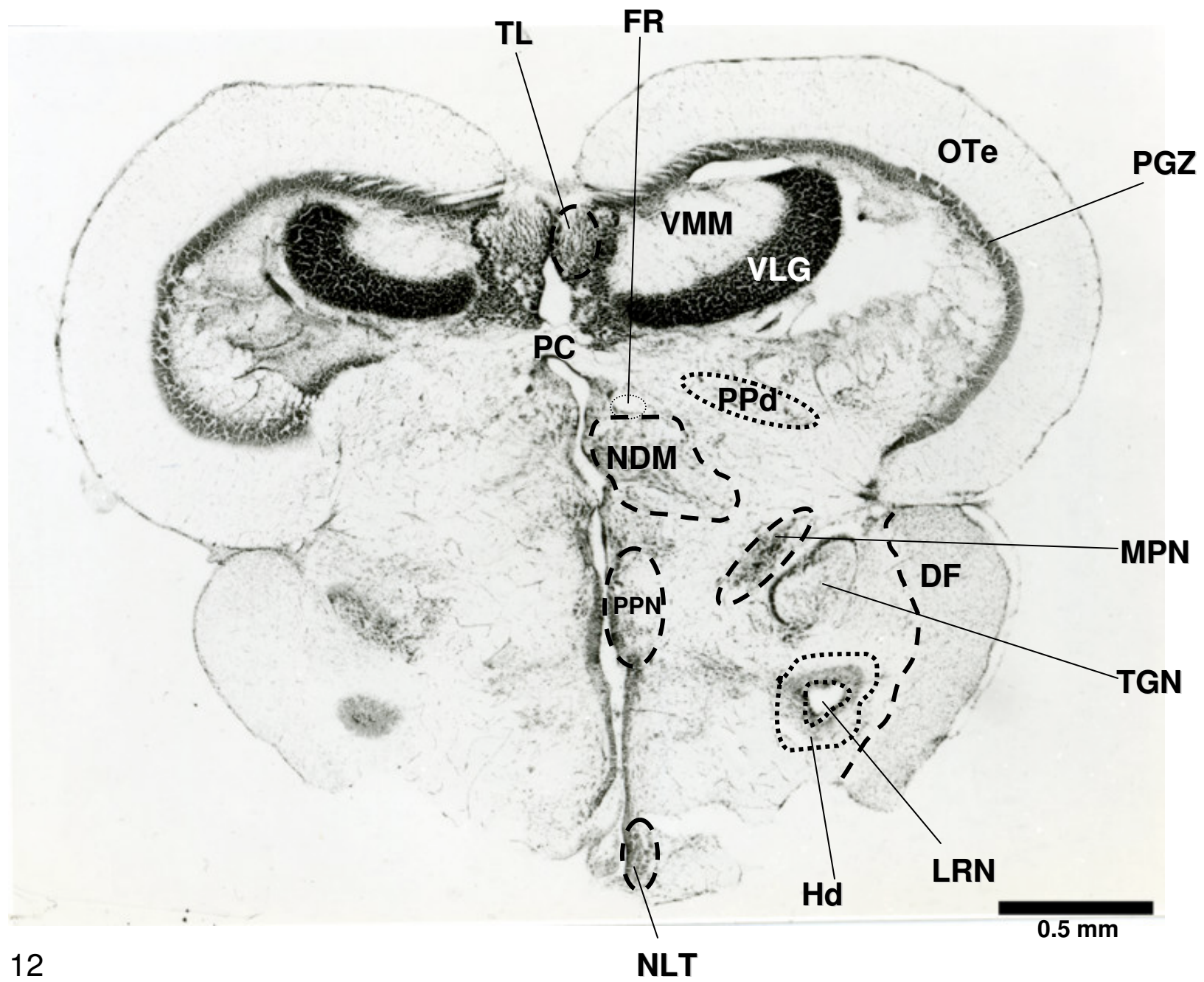
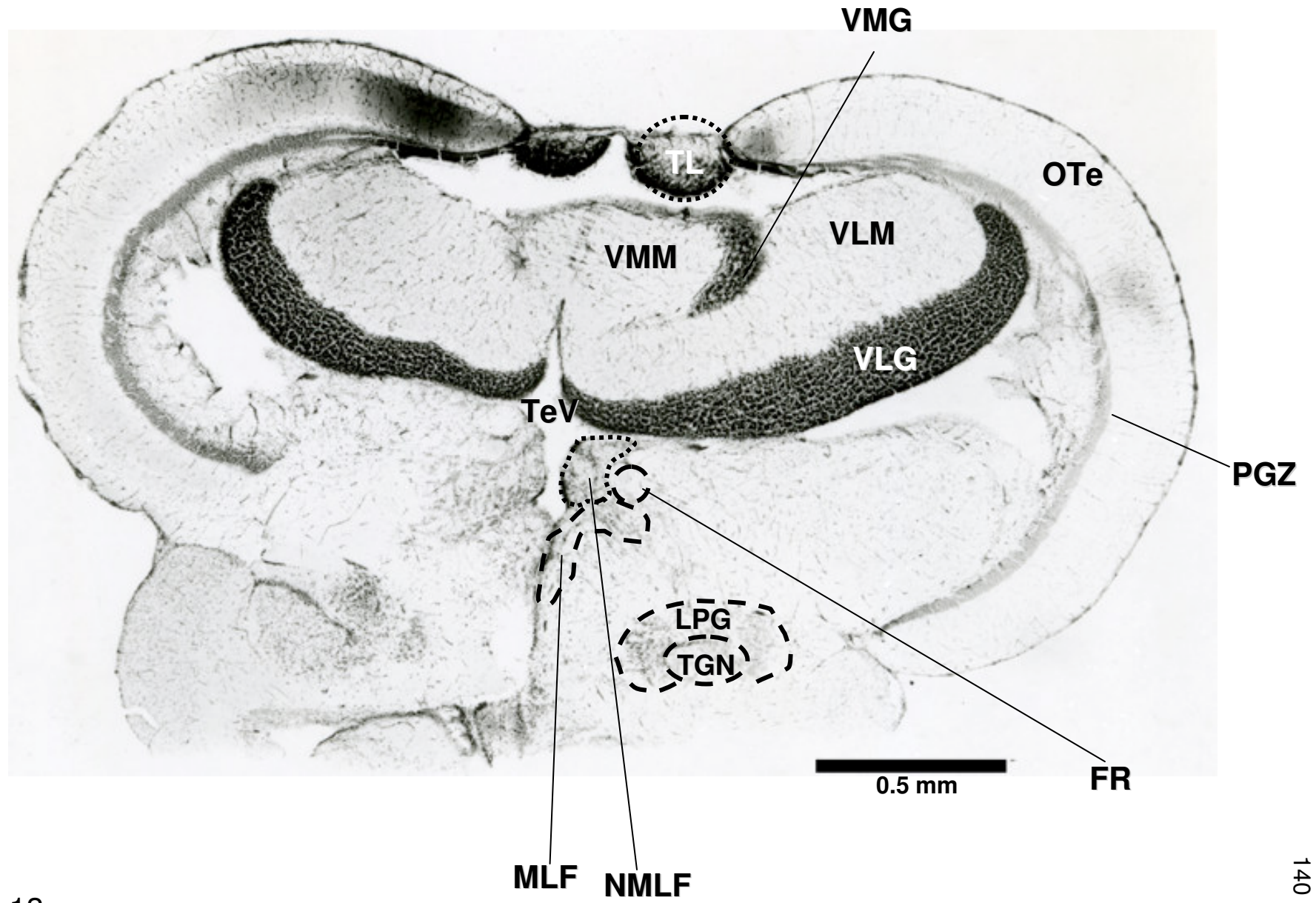
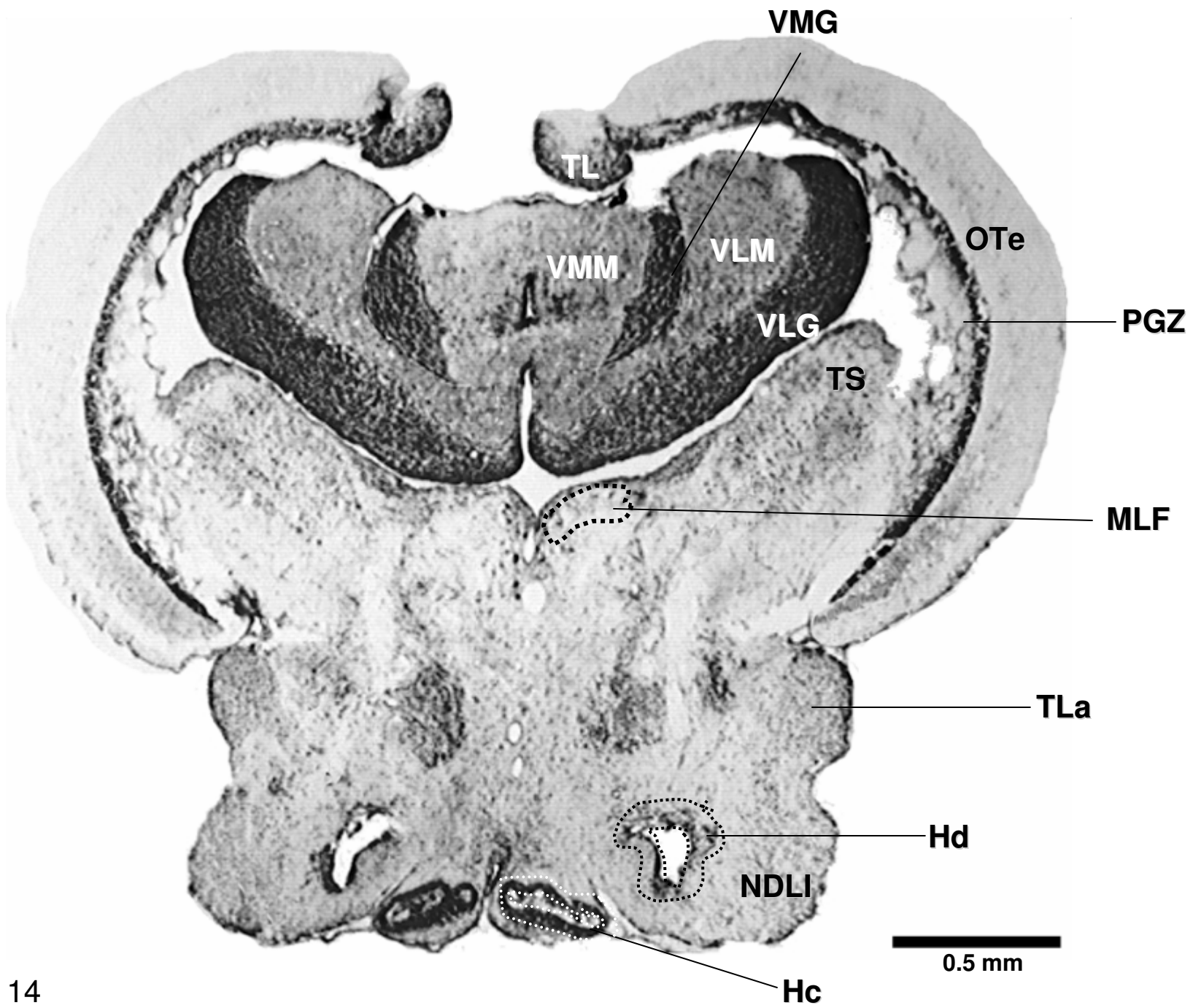


Plate 12





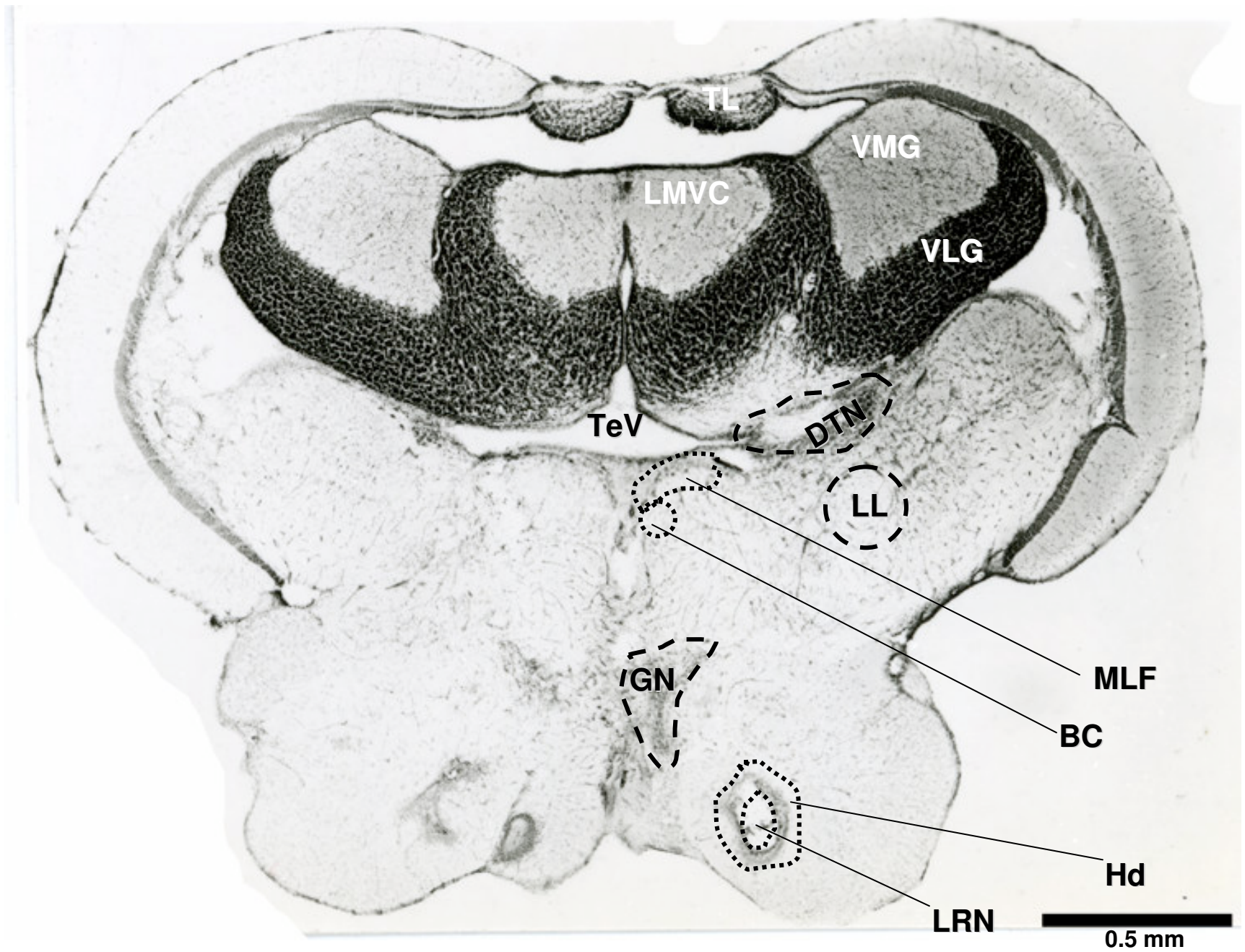
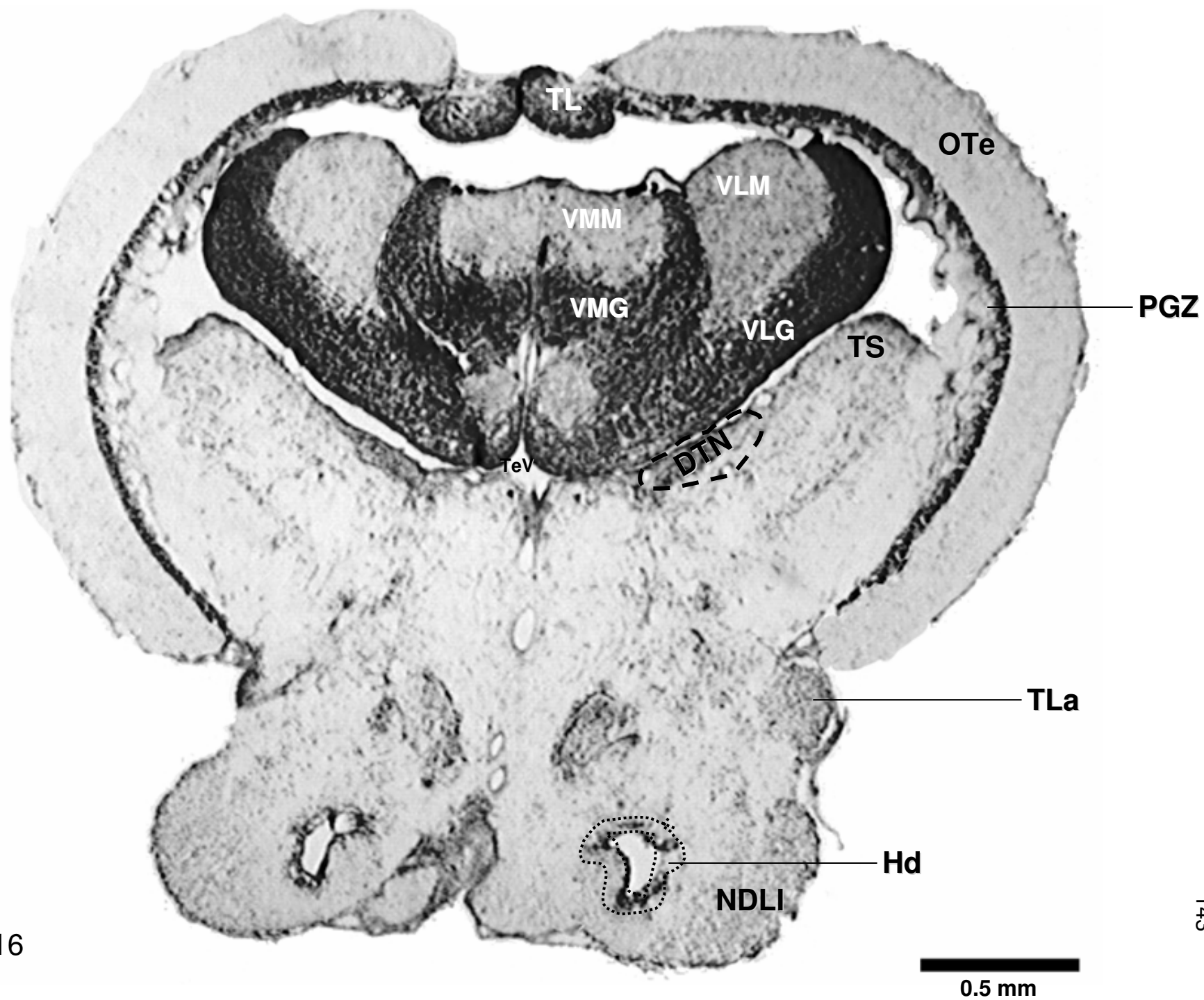
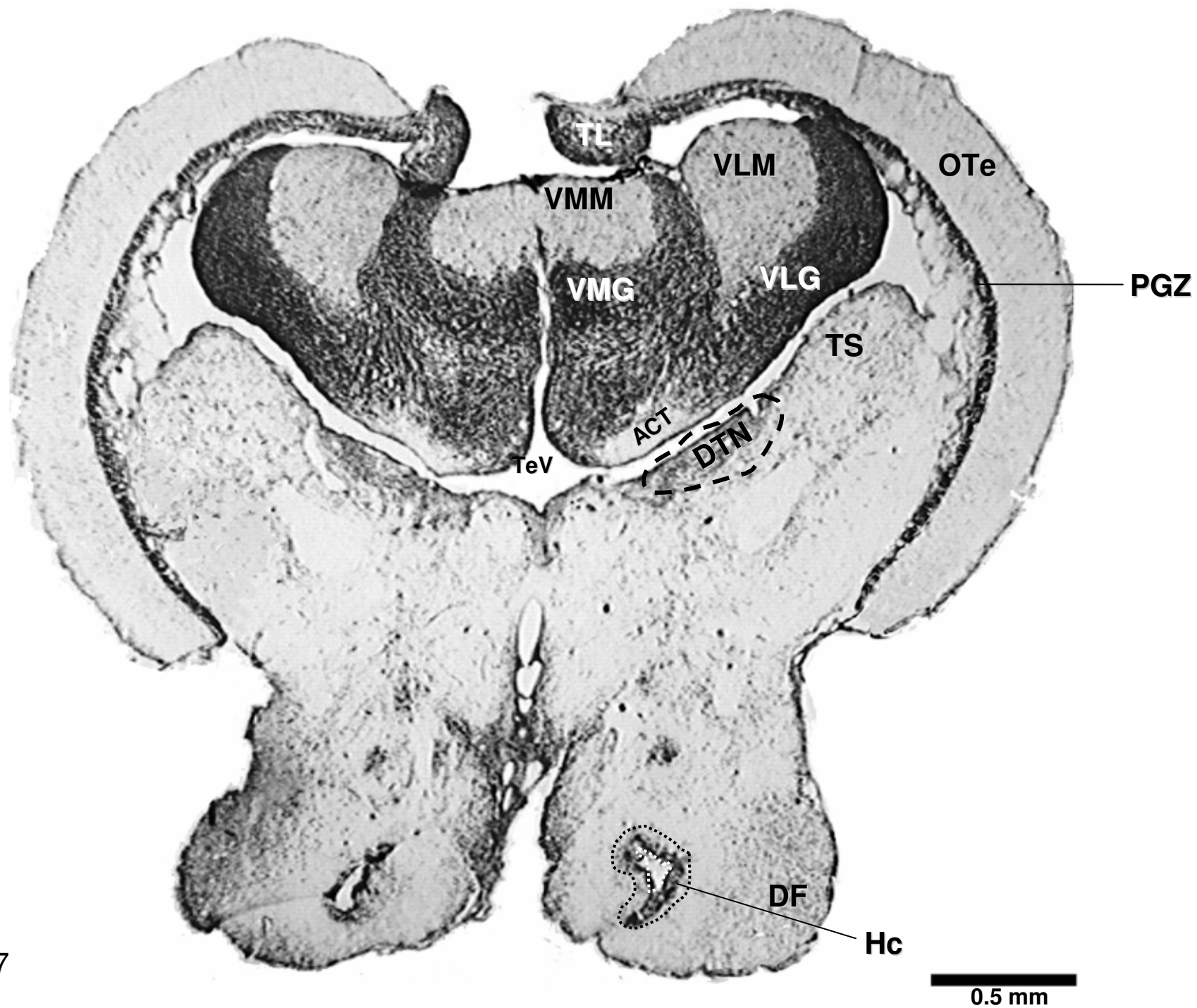


Plate 15





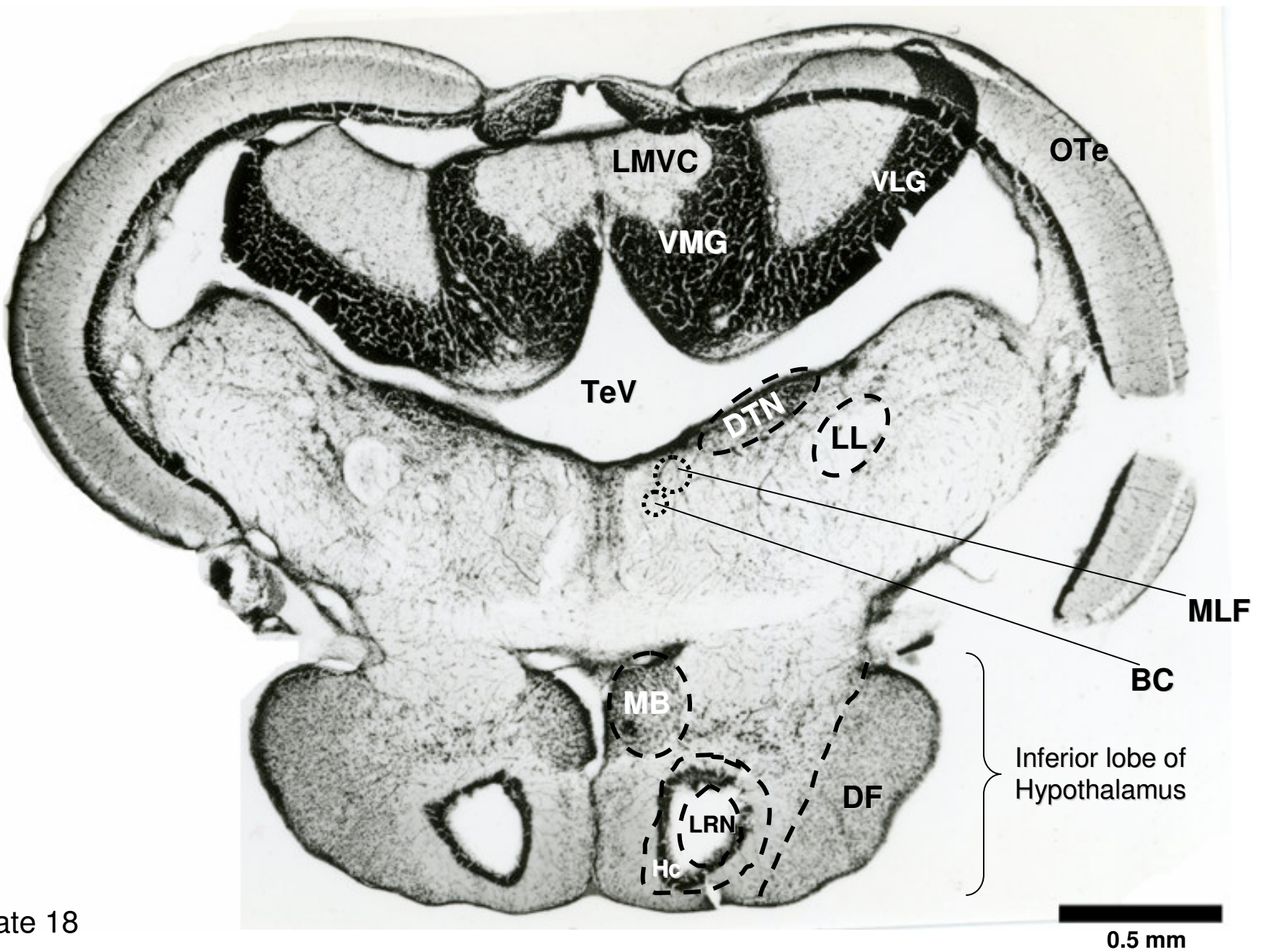
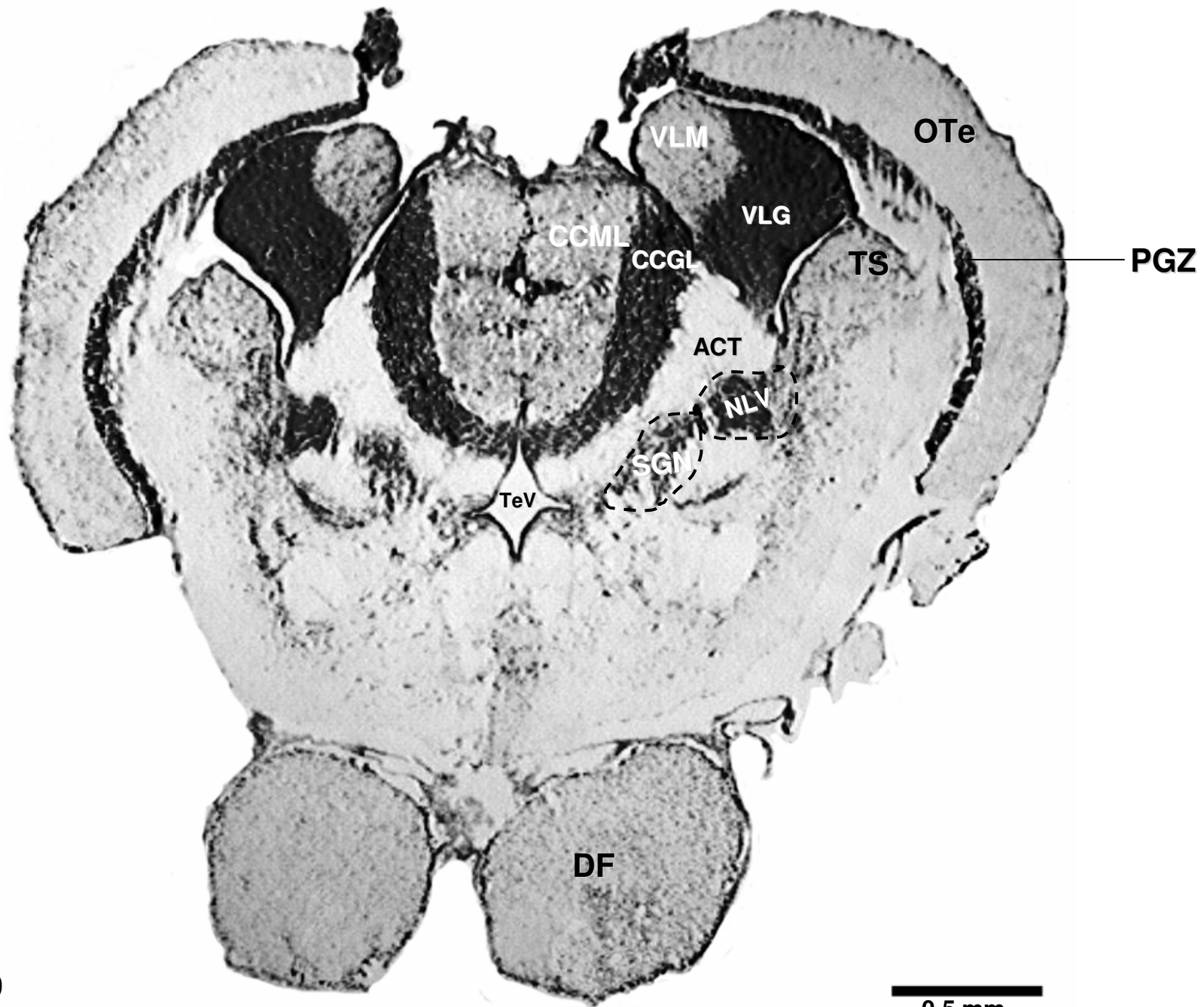
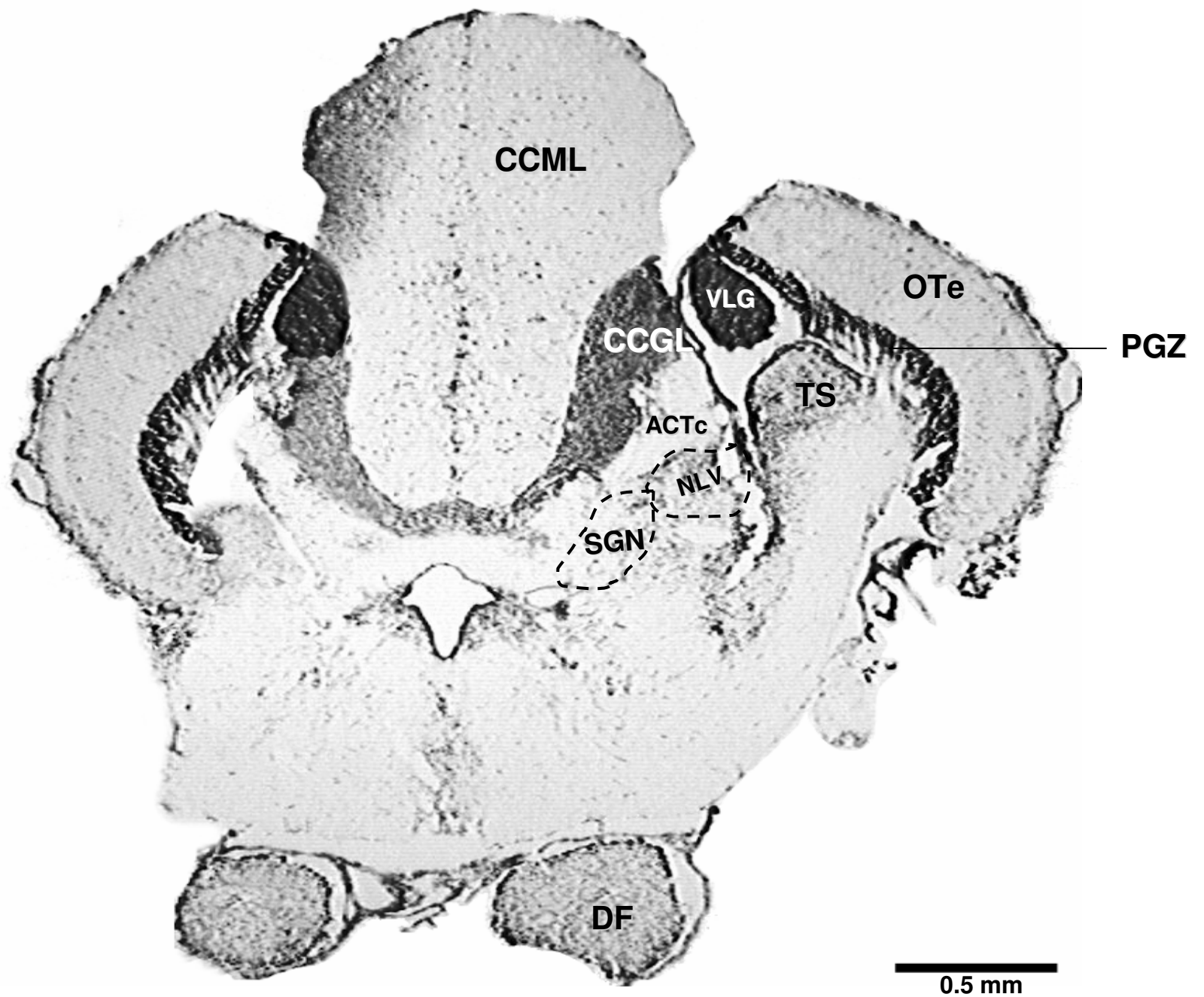
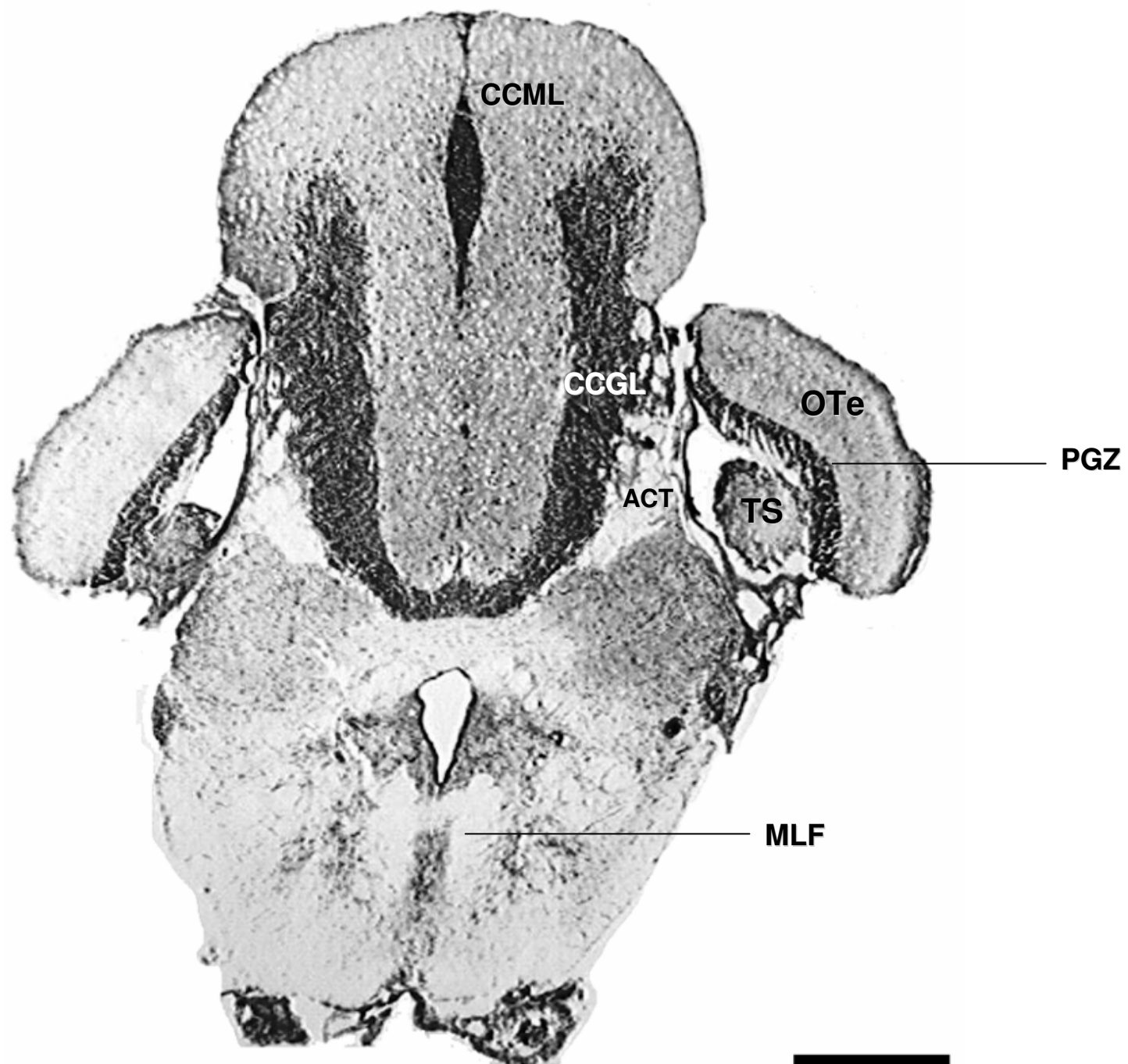
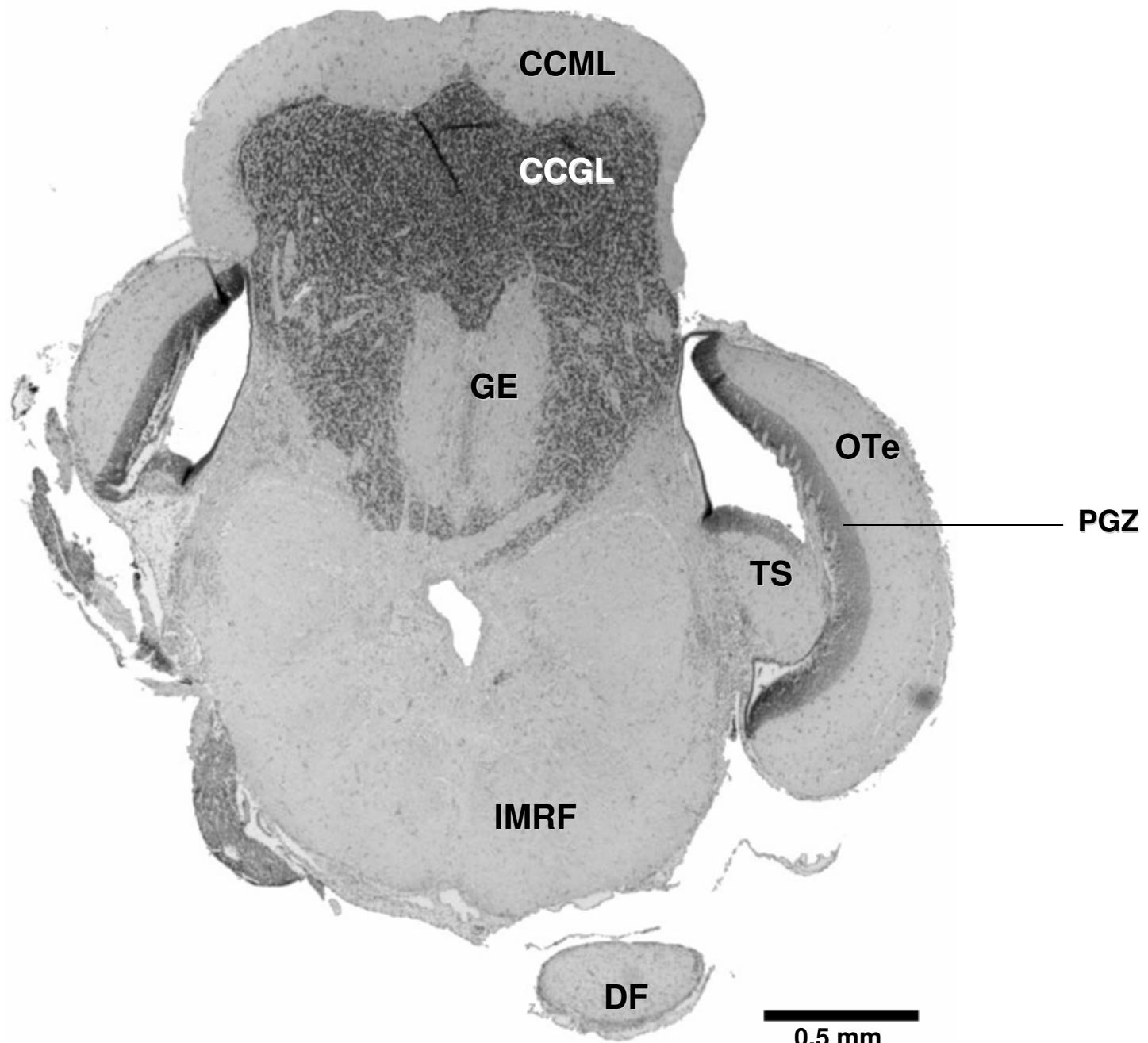


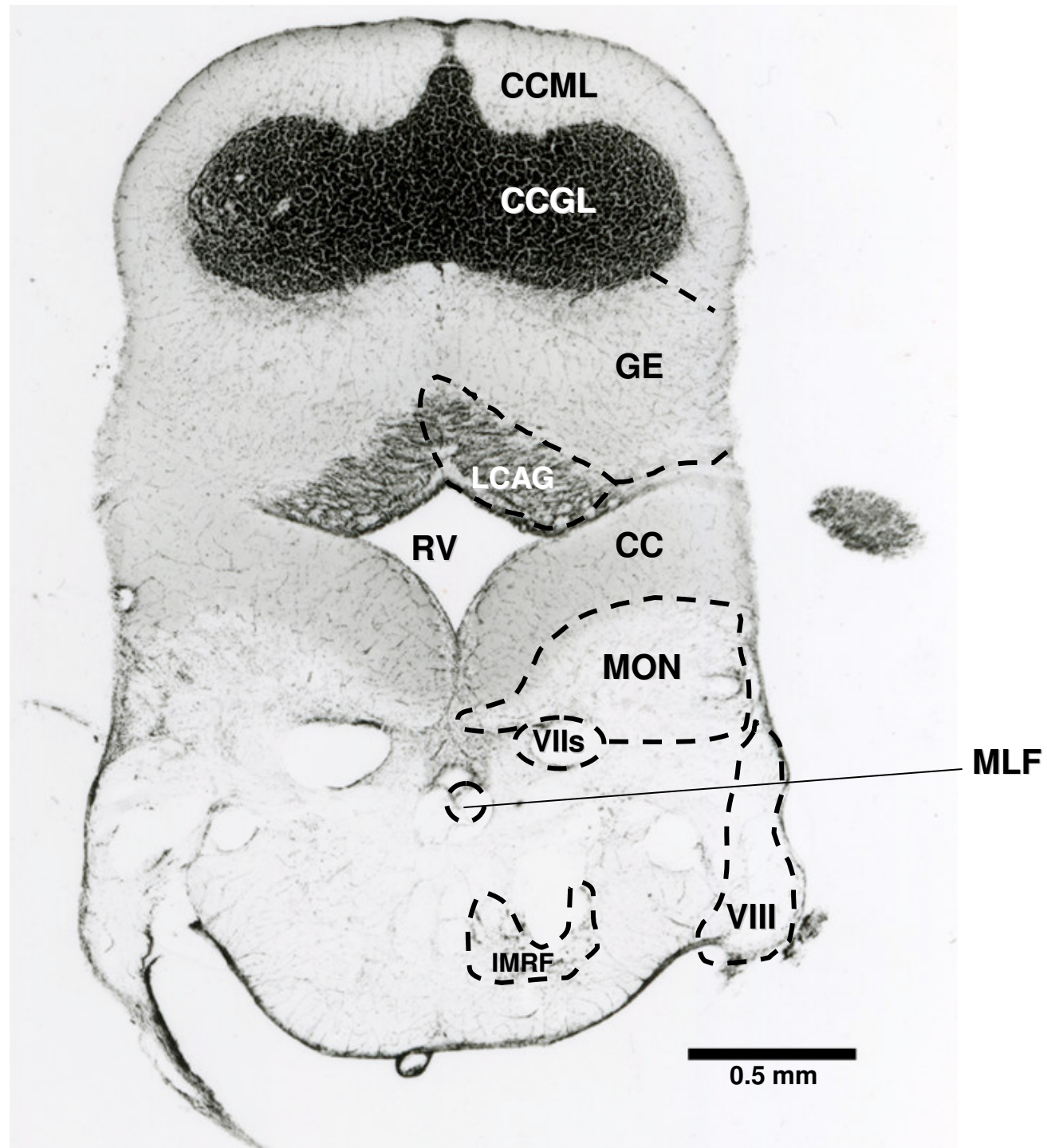
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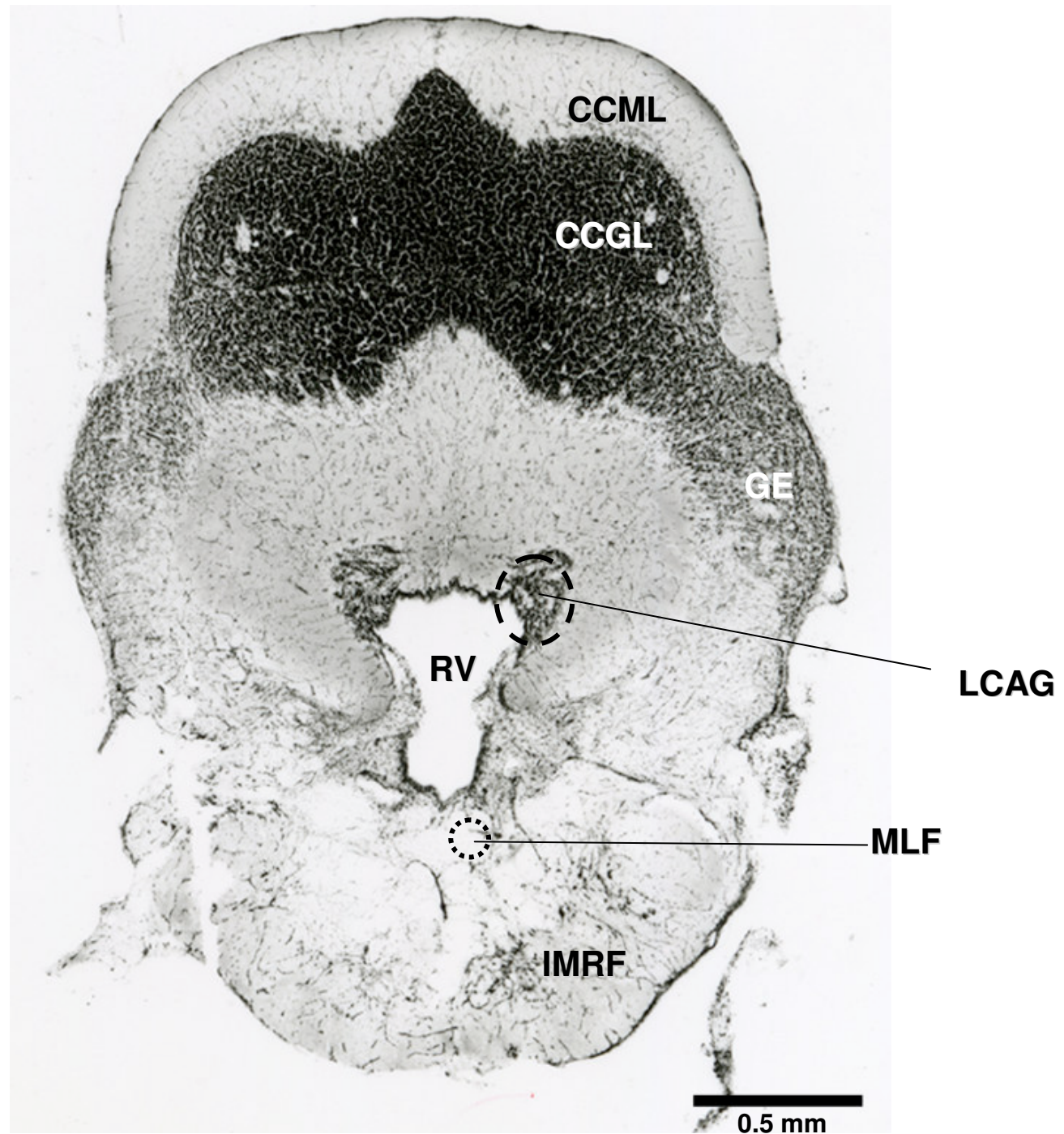


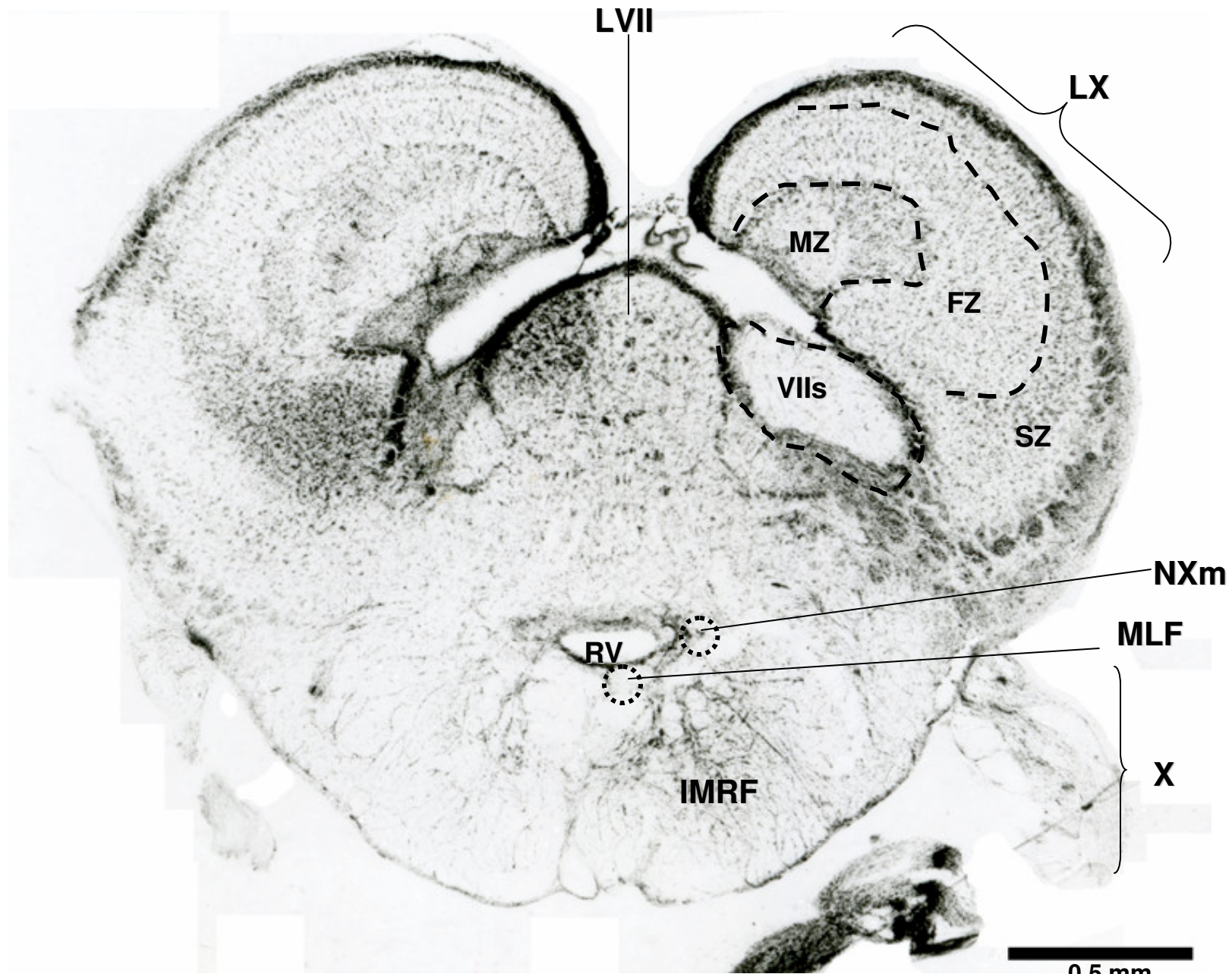


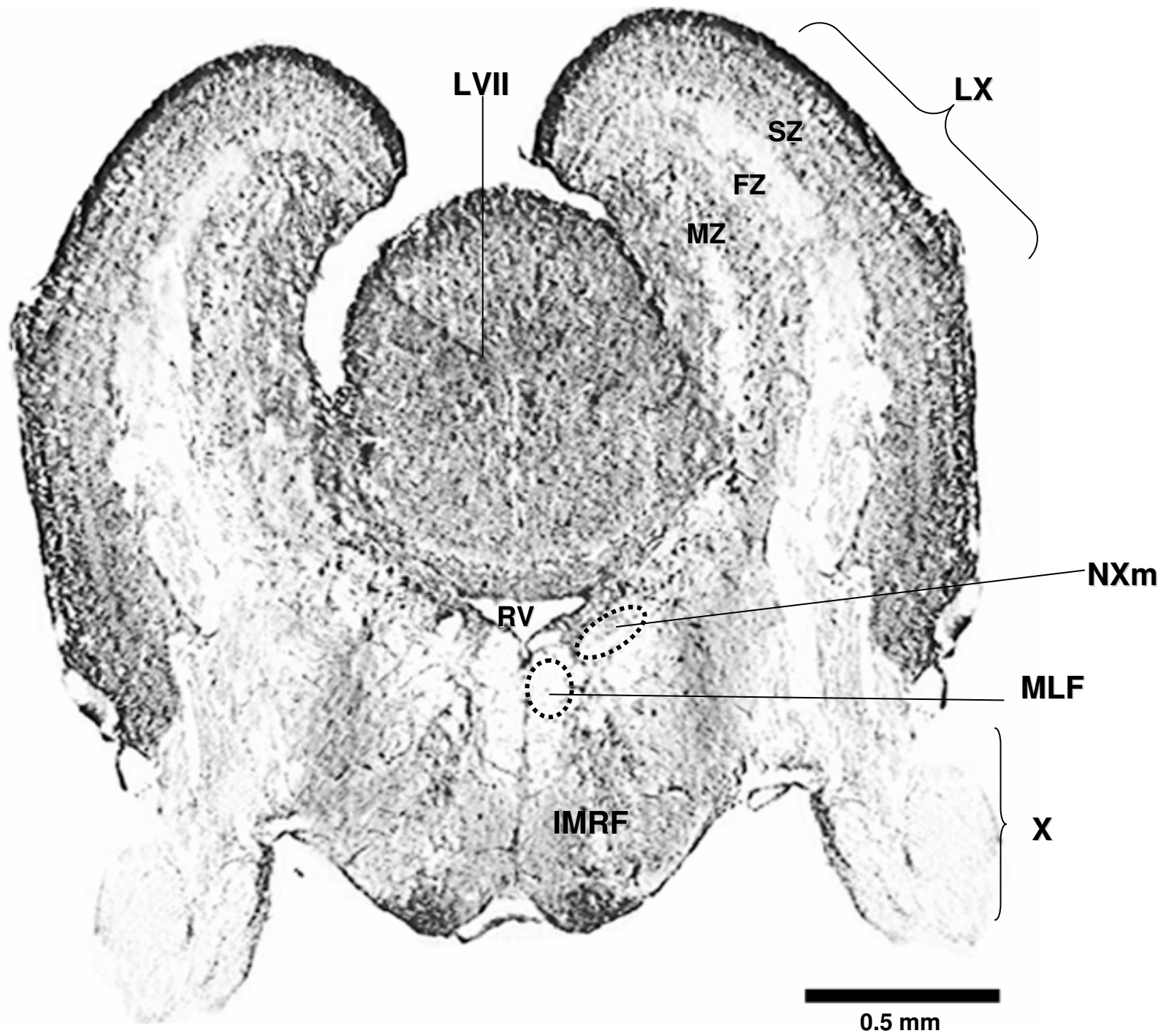


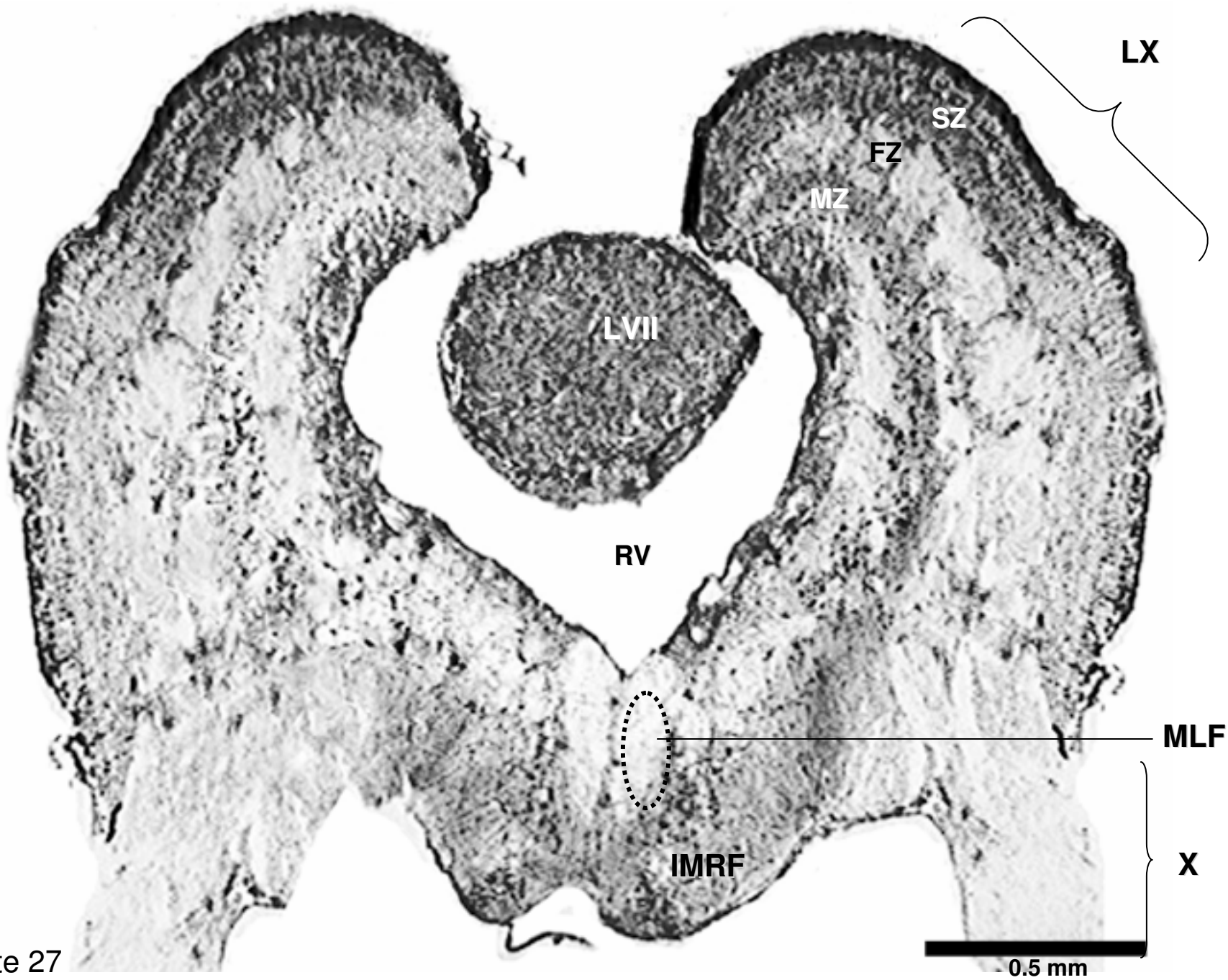


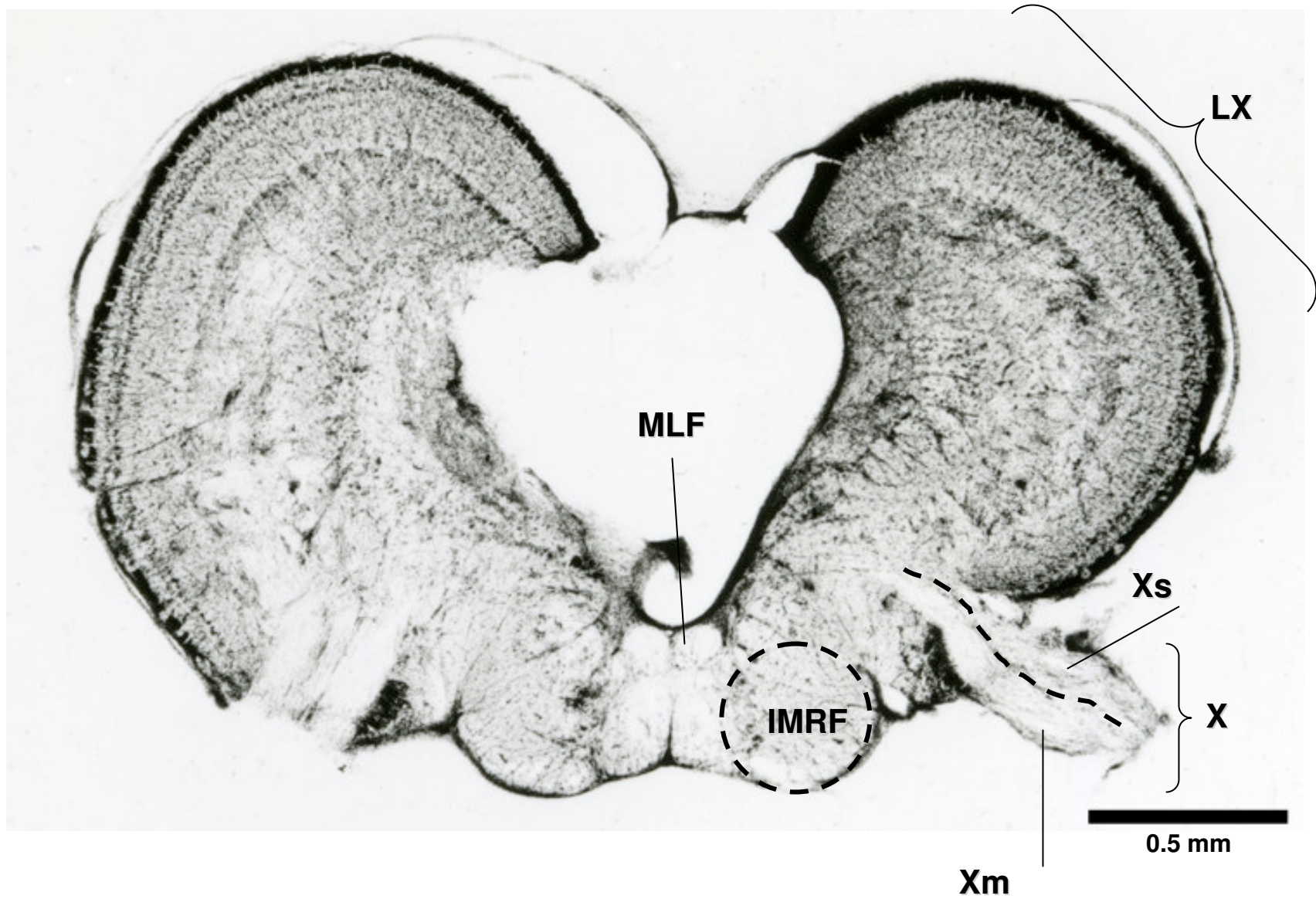


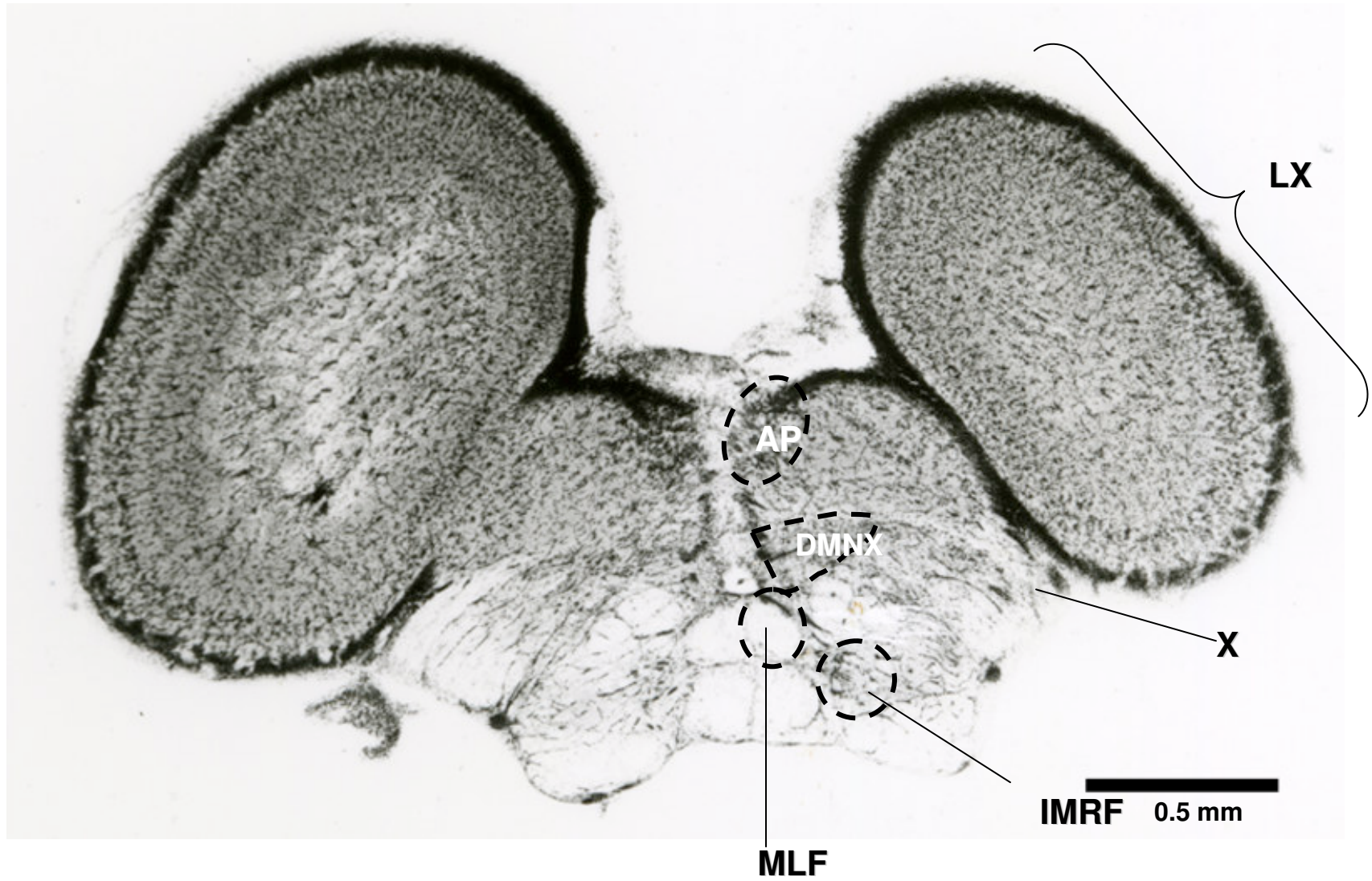












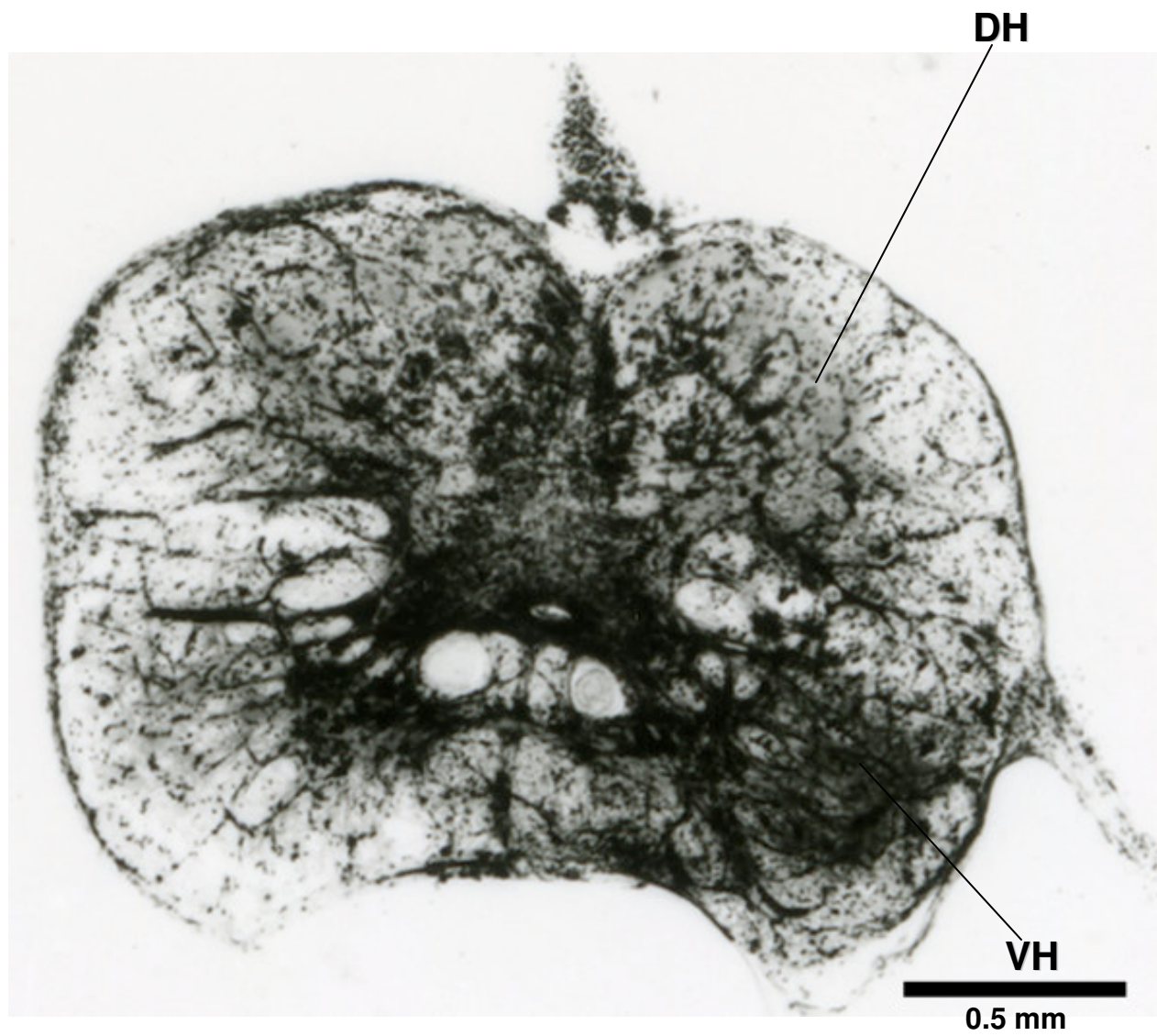


Plate 30

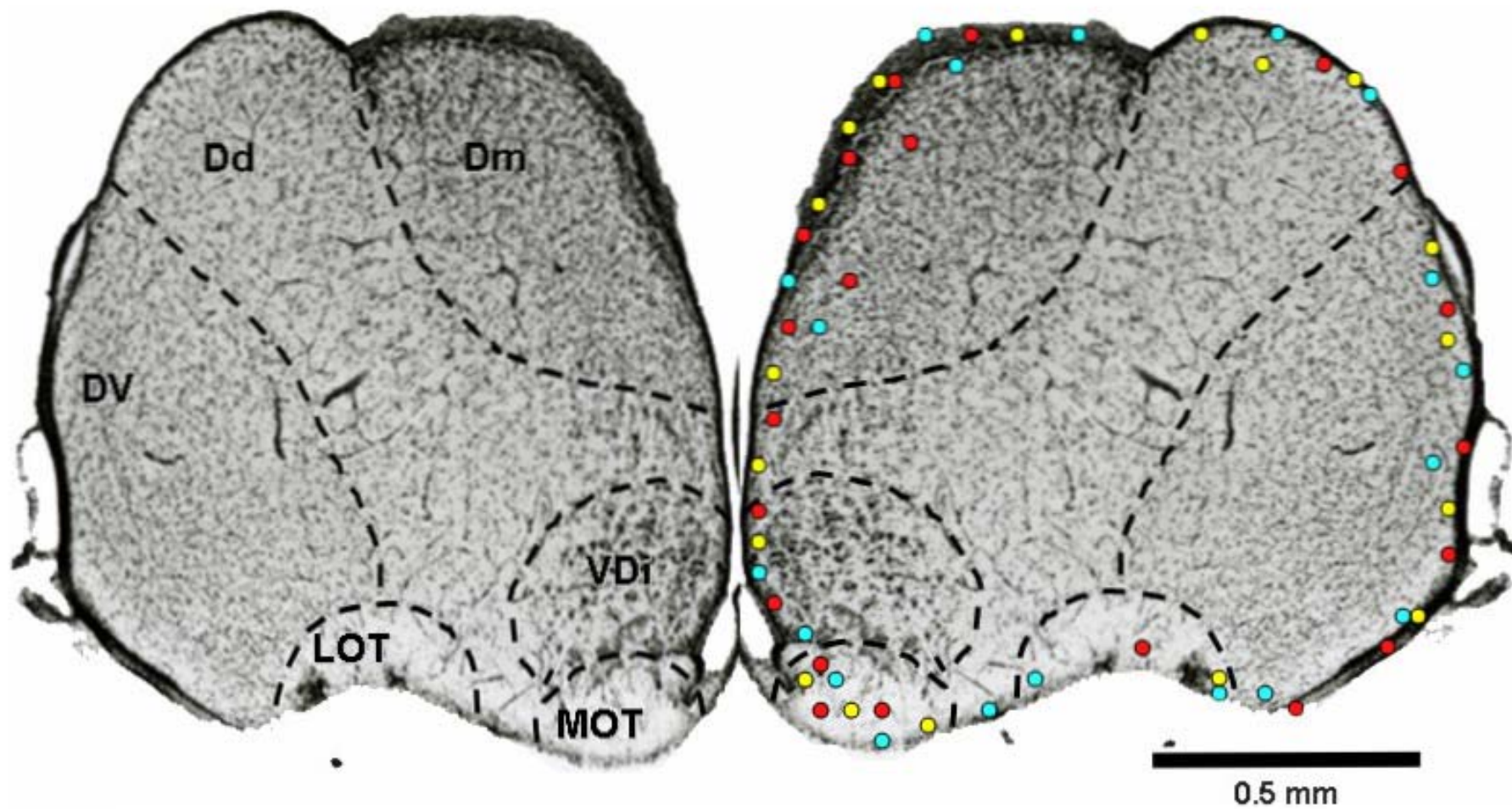
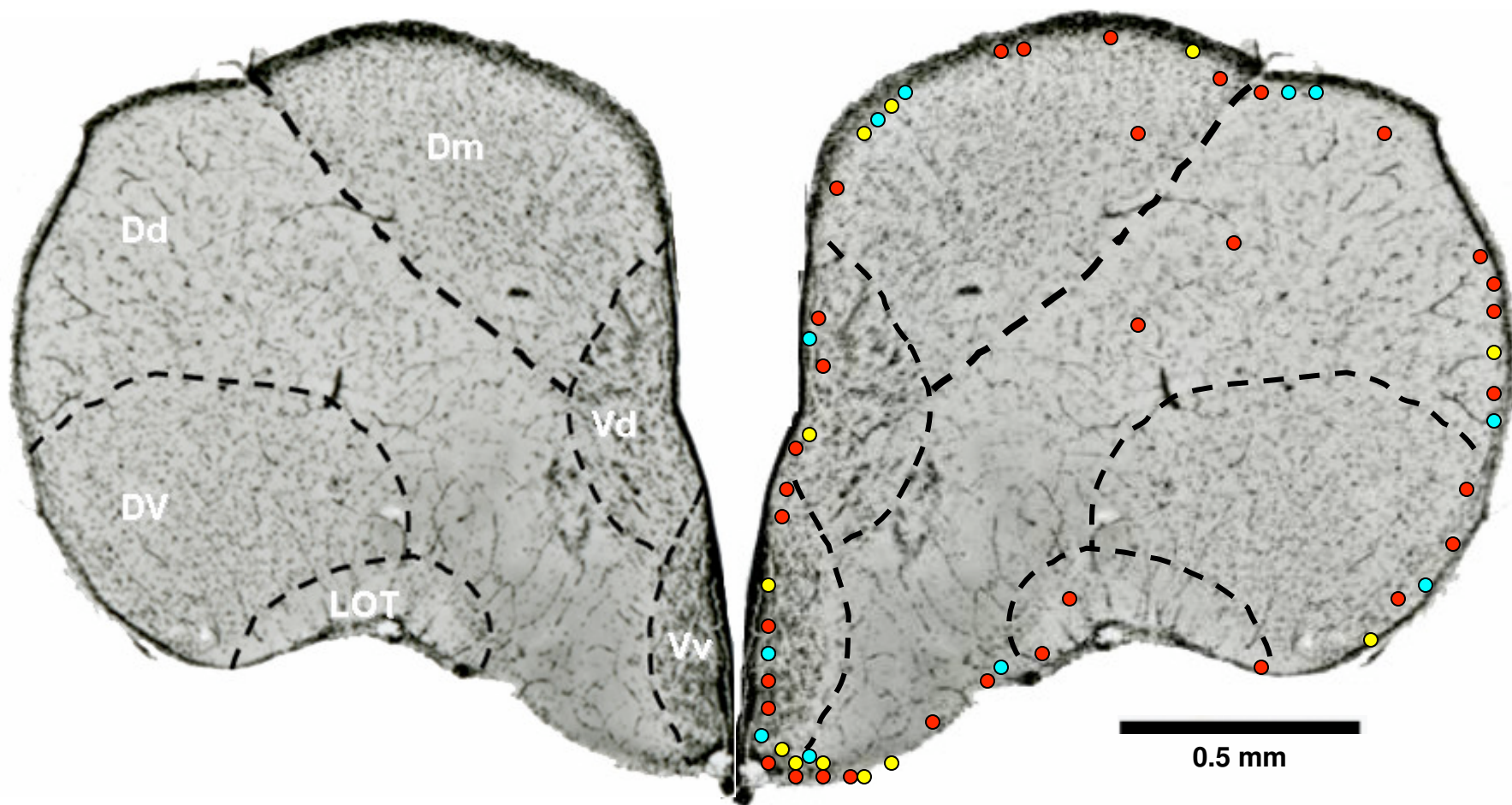


Plate 1



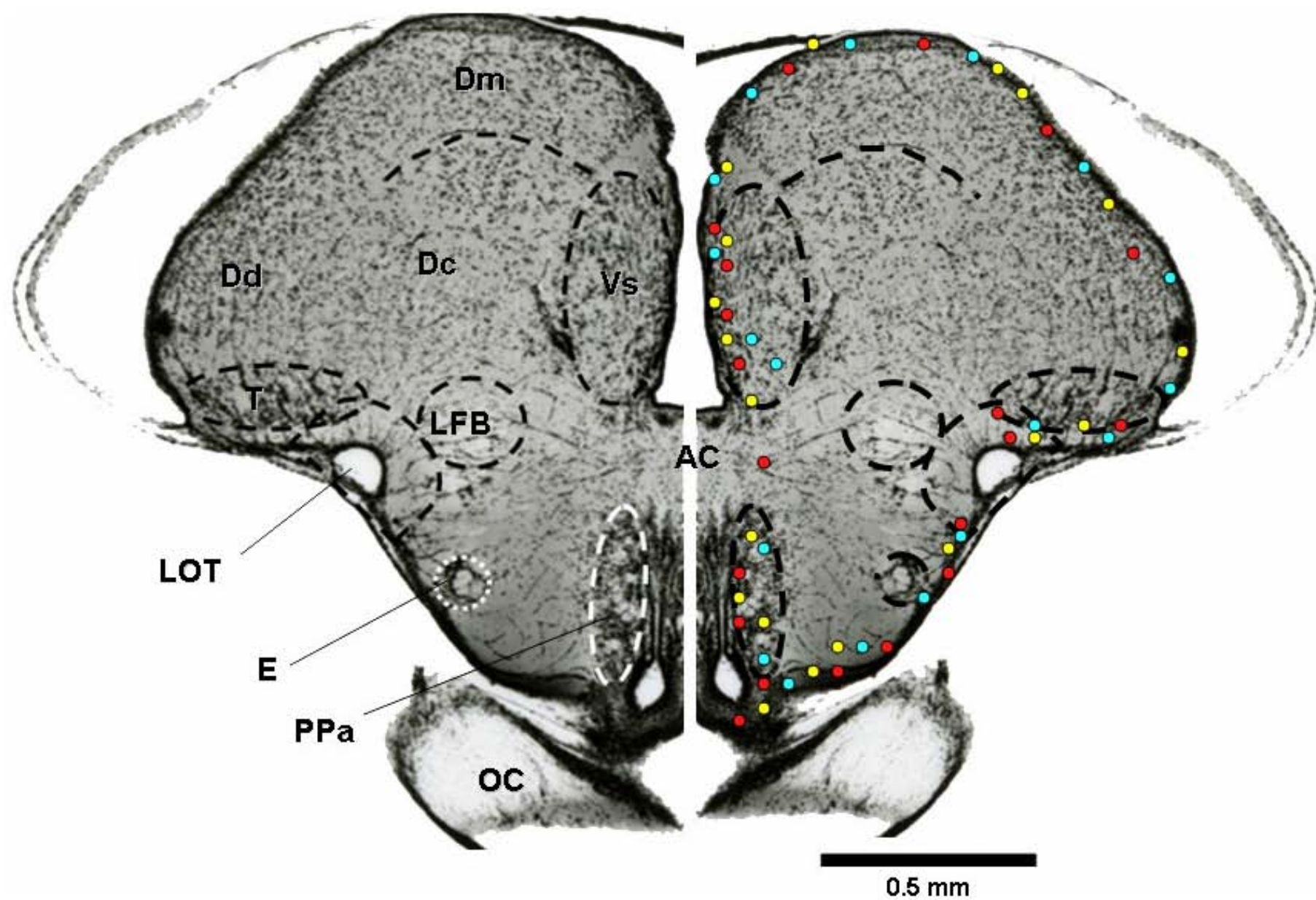


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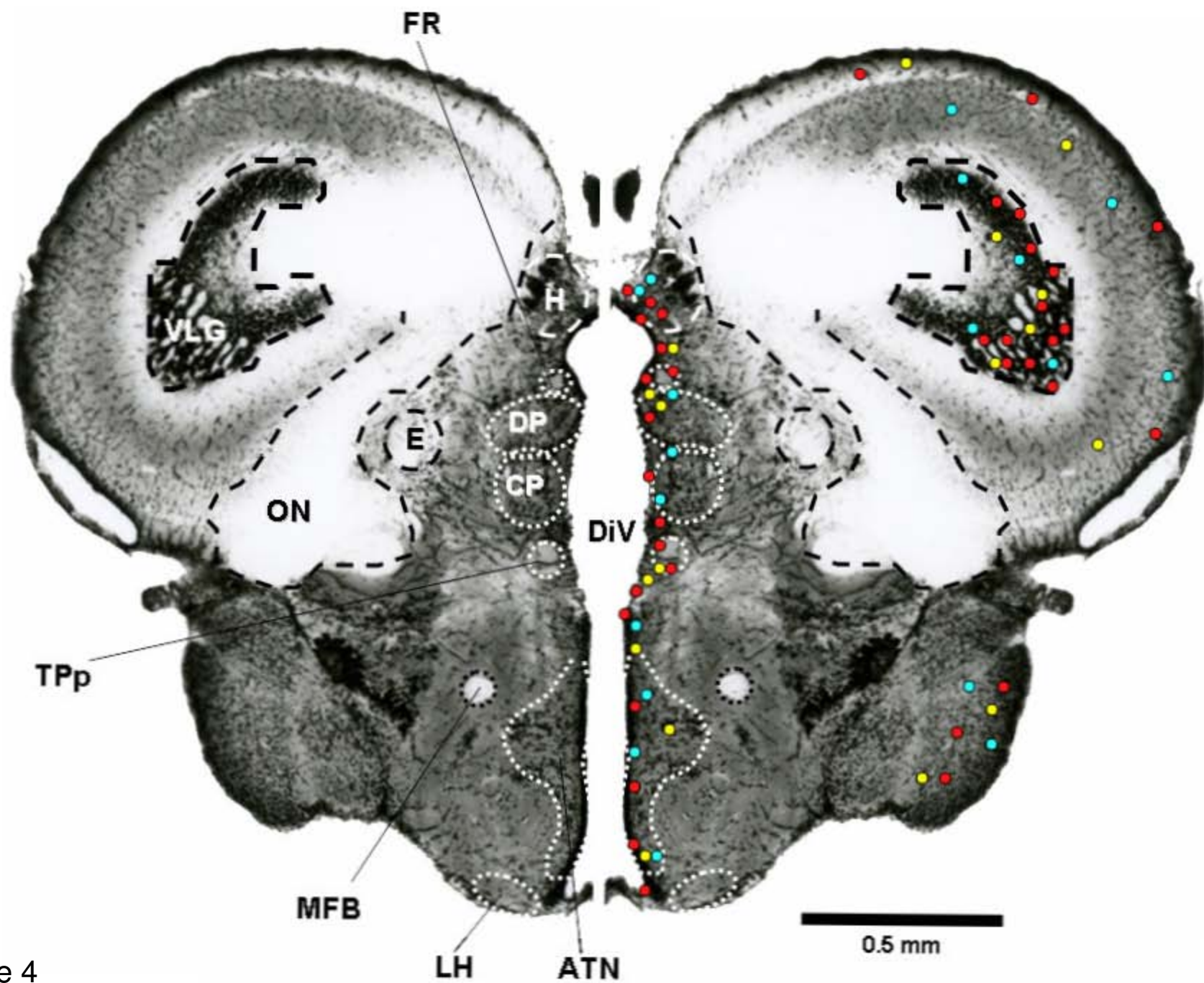


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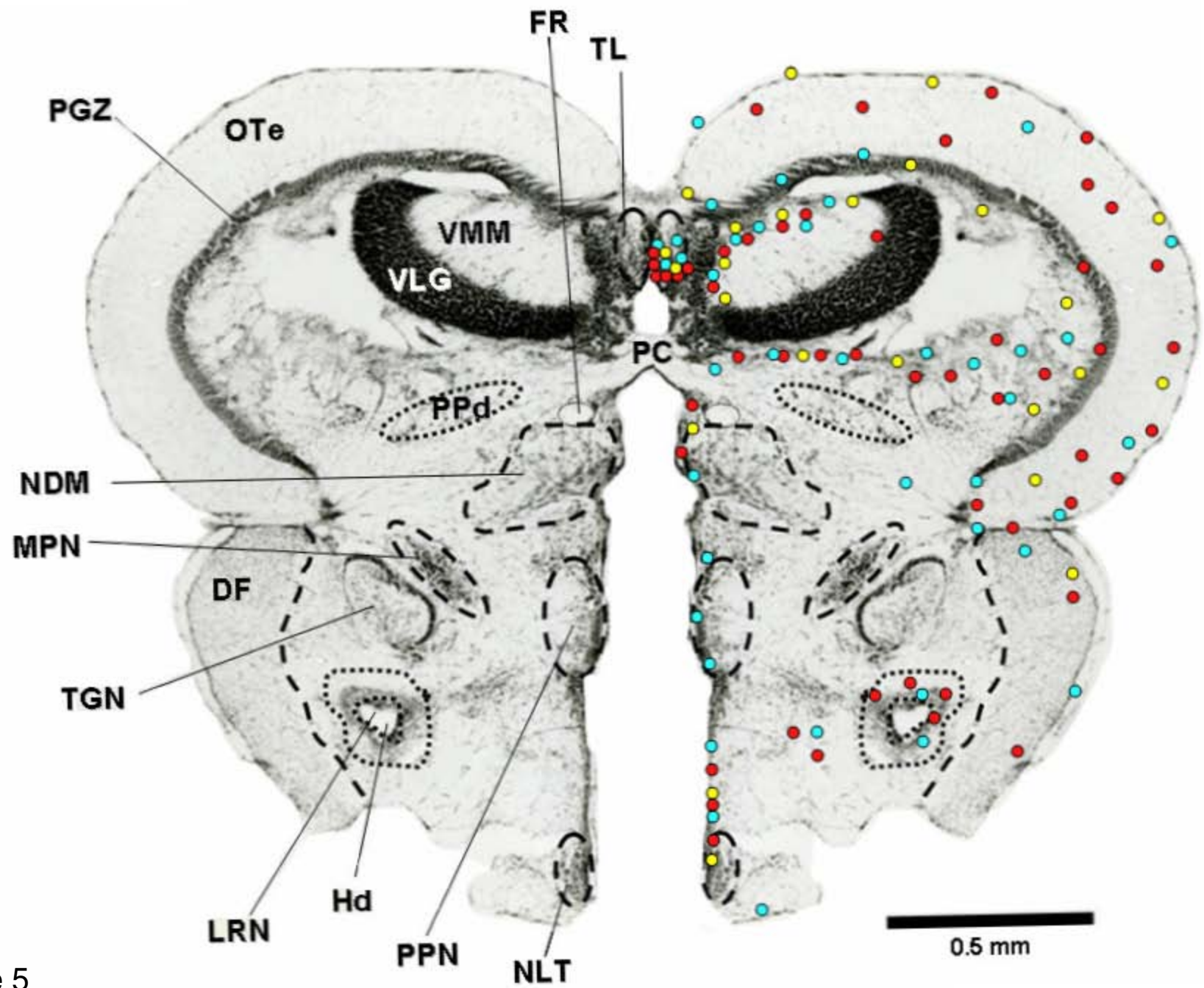


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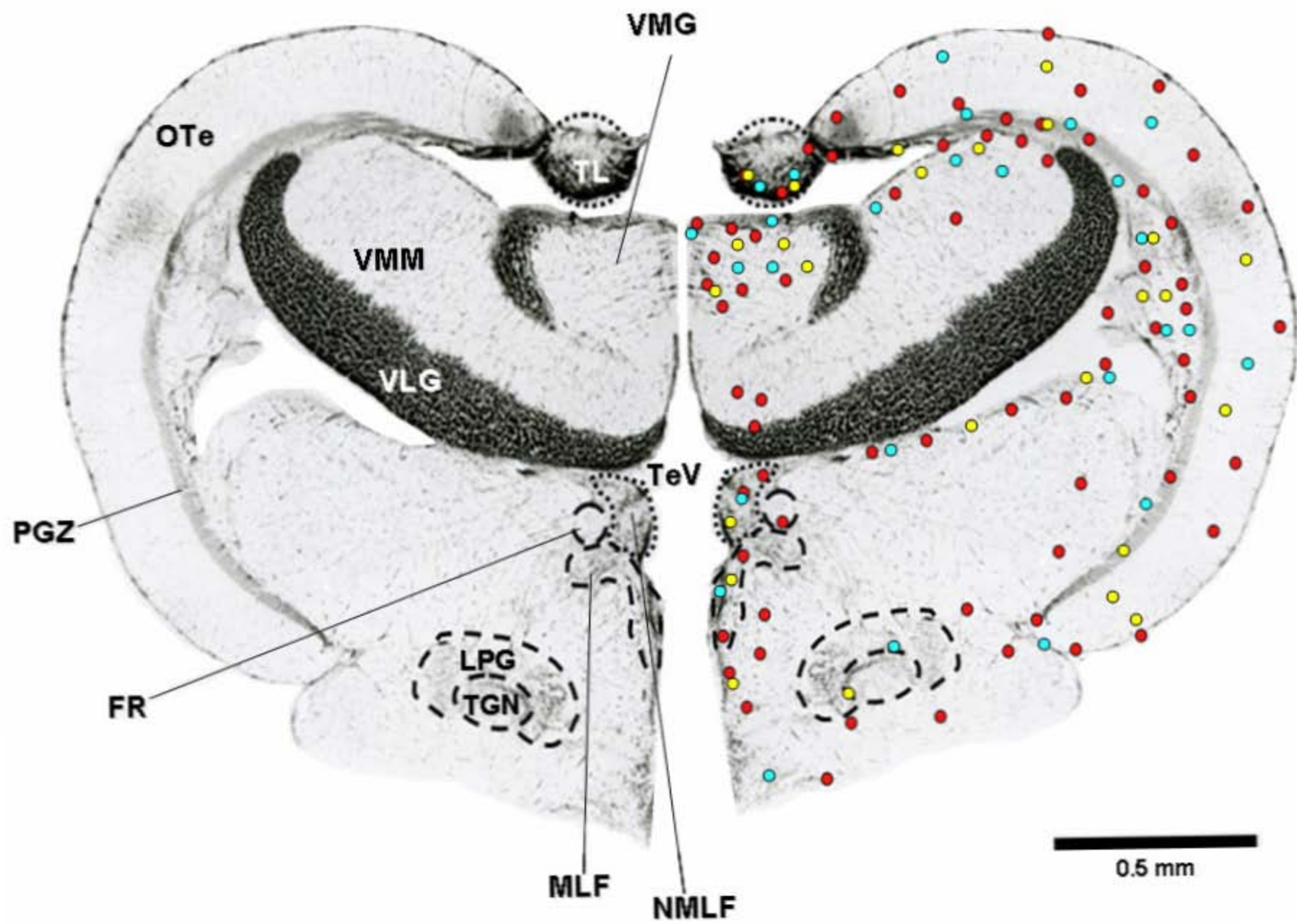


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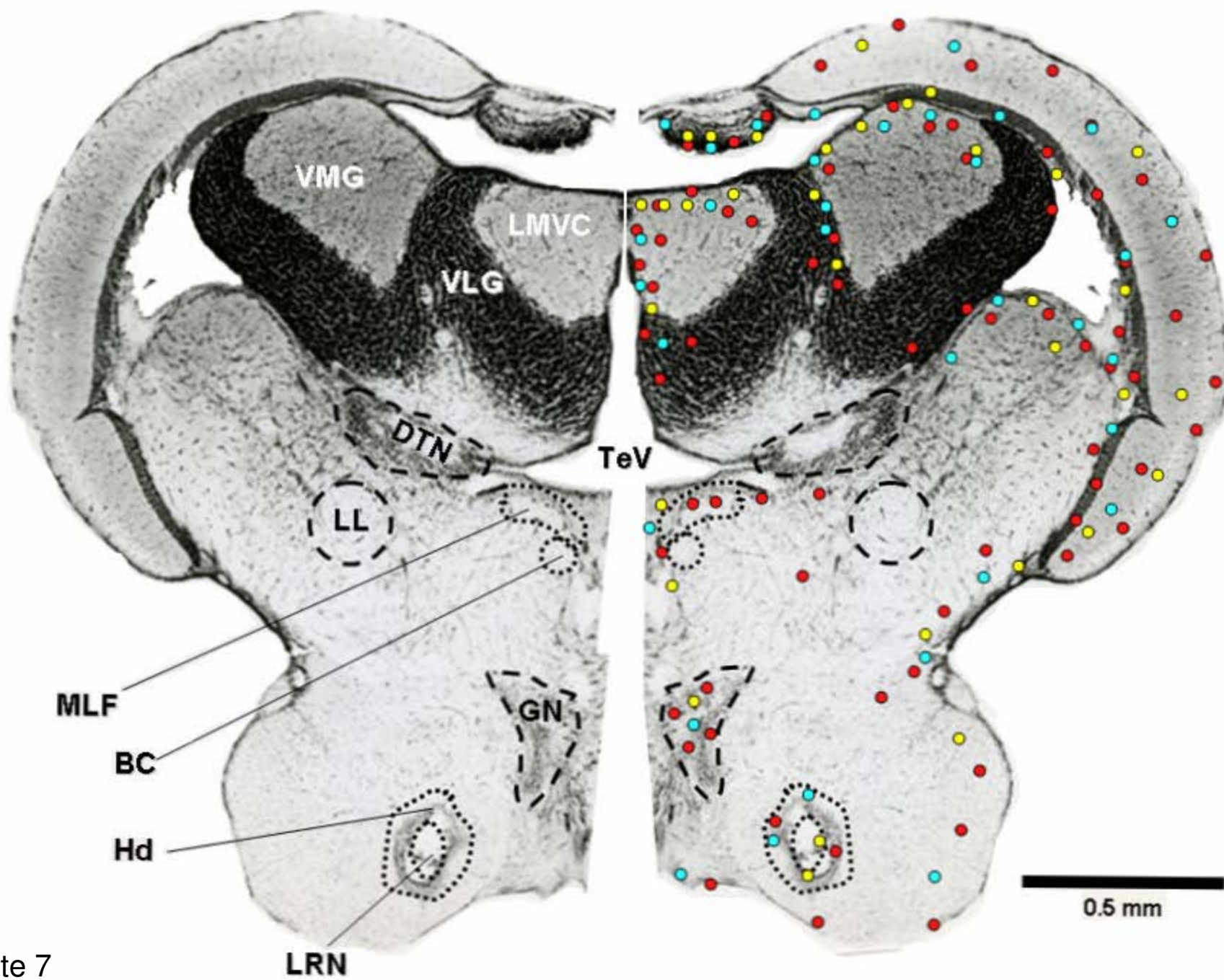


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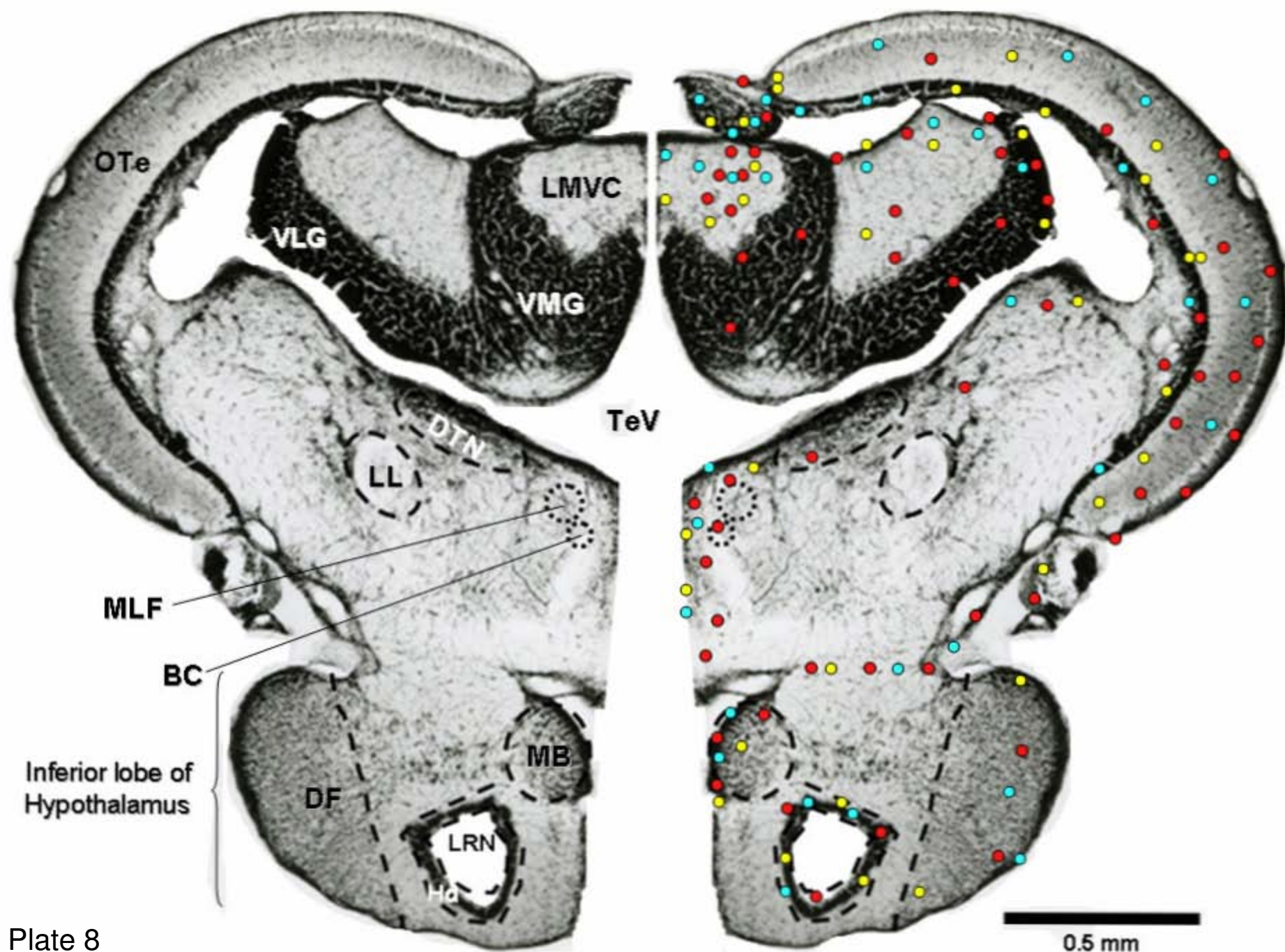


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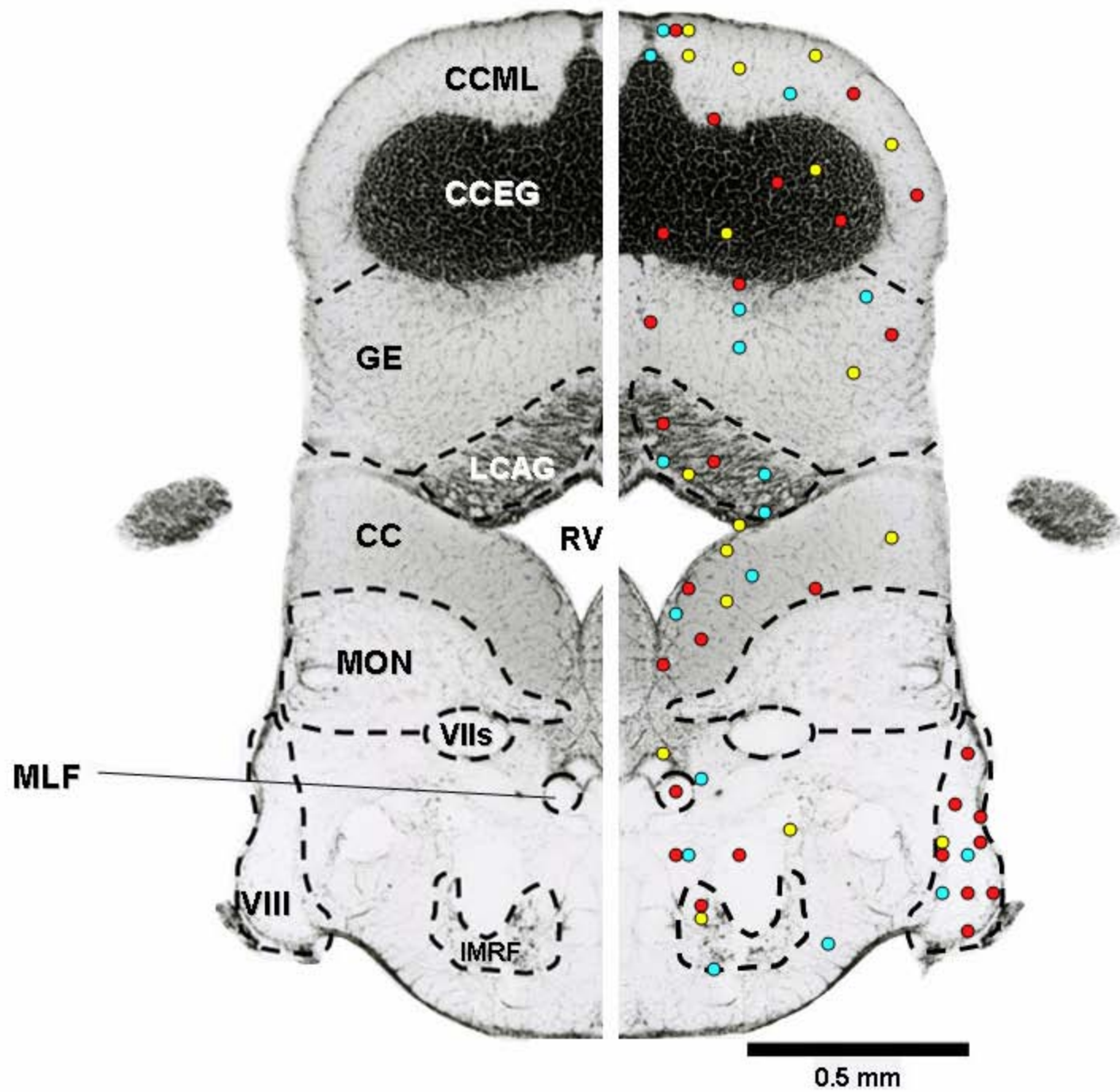


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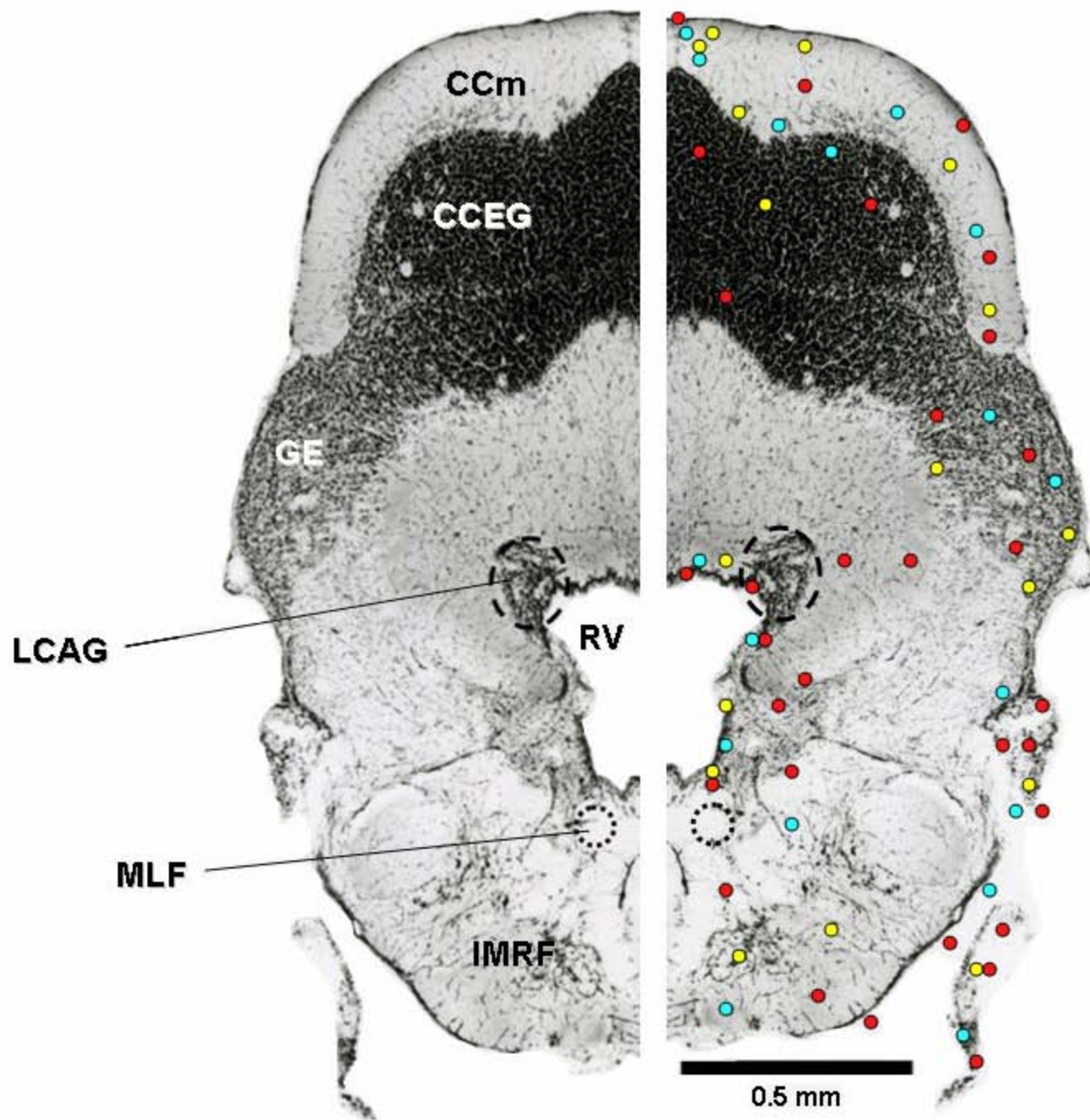


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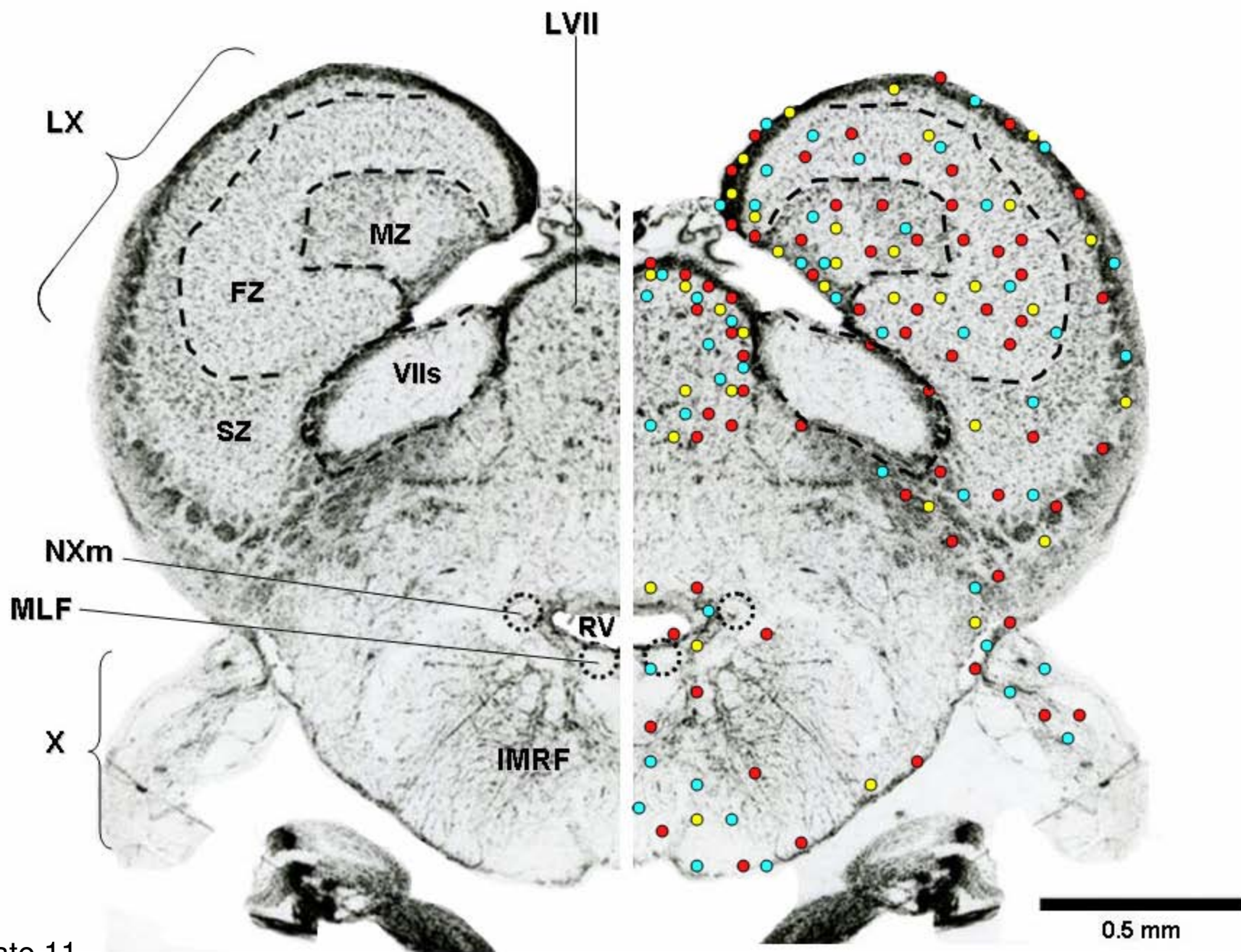


Plate 11

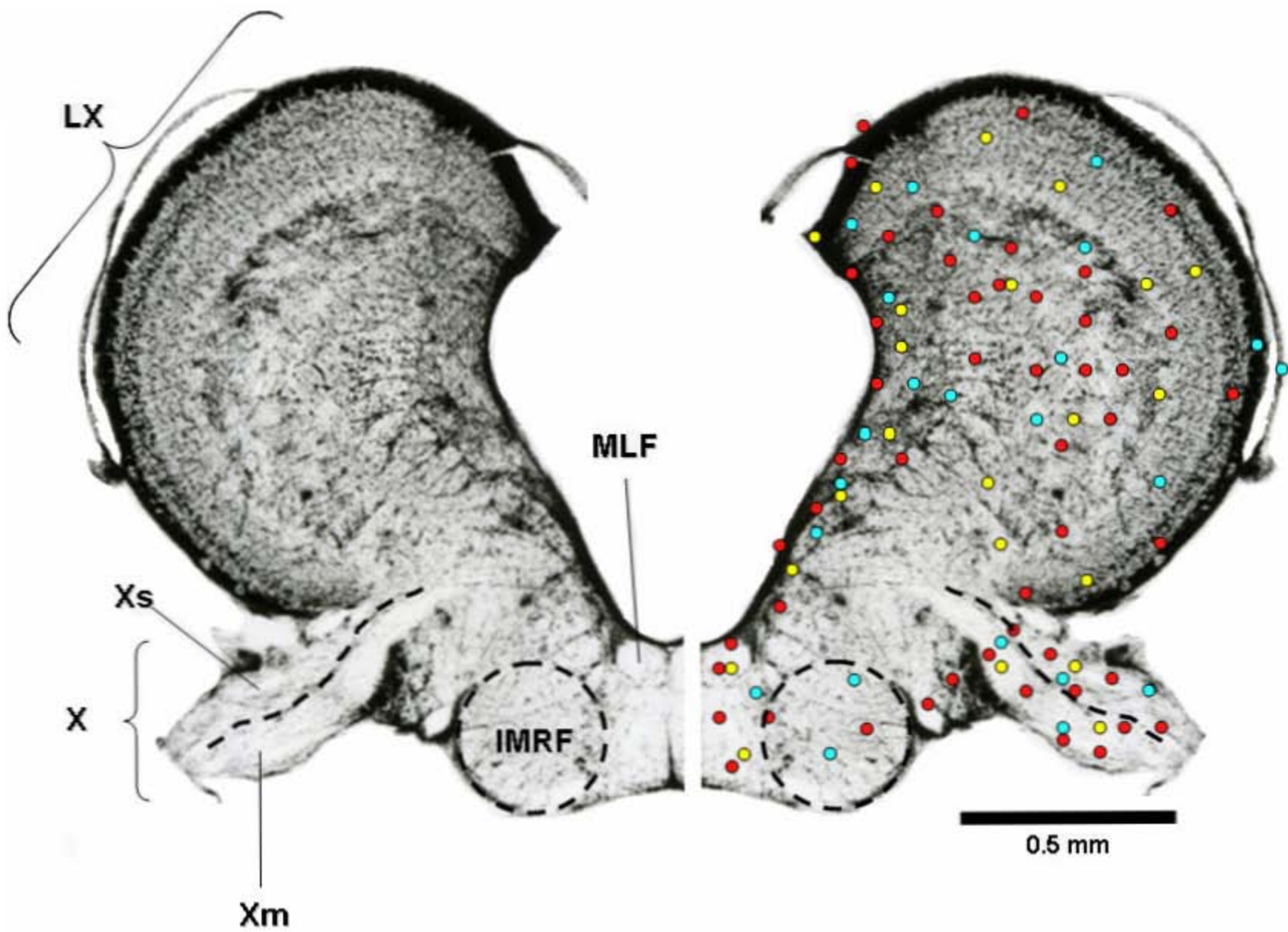


Plate 12