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DEPARTAMENTO DE CIÊNCIAS DA VIDA

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UNIVERSIDADE DE COIMBRA

Mapping the Serotonergic System:

Topographical Organization of Serotonergic Projections from the Dorsal Raphe Nucleus

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Zachary Mainen (Fundaçao Champalimaud) e do Professor Doutor Carlos B. Duarte (Universidade de Coimbra)

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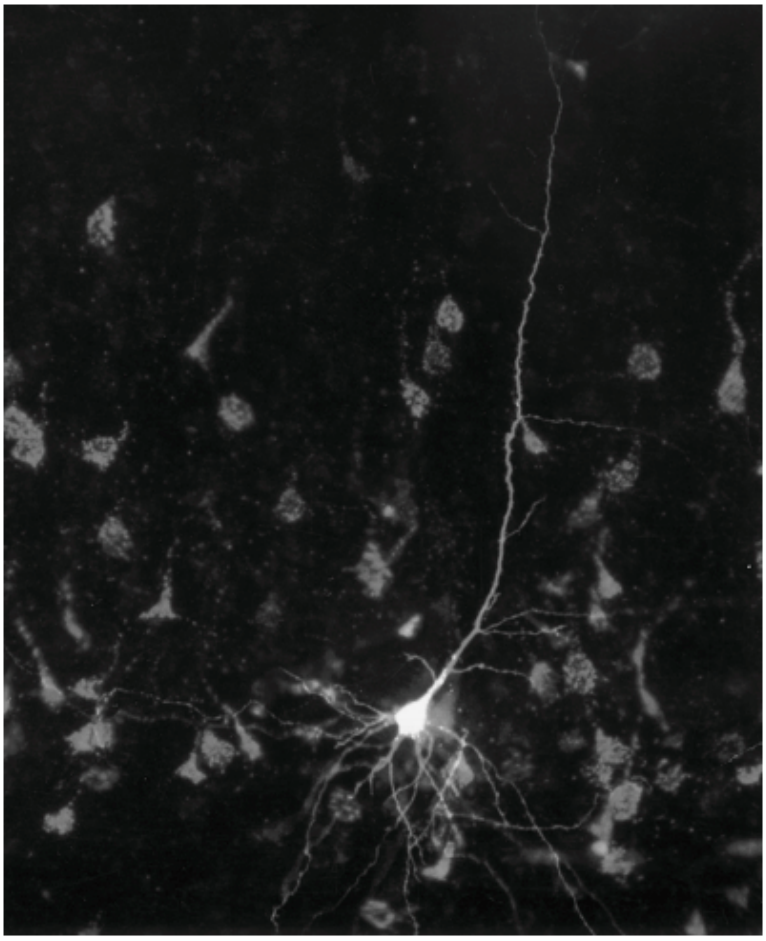
Abstract

The serotonin system is considered one of the most complex neuromodulatory systems in the central nervous system and is one of the most frequently targeted systems for pharmacological studies addressing a wide range of psychiatric dysfunctions. Serotonergic neurons are topographically organized with anatomically distinct groups receiving synaptic input from specific brain structures, suggesting organization in both function and projection output to a wide range of forebrain and brainstem circuits. There still remains much confusion about the role of serotonin, and many of the functions effected by serotonin modulation, including affective control, behavioral inhibition and sensorimotor gating, may be linked to the structural organization mapped within the system. The aim of this project is to develop and implement labeling methods to visualize and control specific projection pathways of serotonin neurons originating in the dorsal raphe nucleus. Retrograde labeling tools were explored and optimized. We were able to optimize the experimental protocol for retrograde labeling using latex microspheres, and the results confirmed that there are projections from the dorsal raphe nucleus to both the piriform cortex and the olfactory bulb with a majority of these neurons being serotonergic. Piriform cortex projecting cells were more numerous and clustered in the posterior portion of the dorsal raphe region. Two types of retrograde viruses were also tested, though with less success and efficiency. They do, however, offer future possibilities for research exploring the structural and functional anatomy of the serotonin system.

KEYWORDS: Serotonin, 5-HT, dorsal raphe nucleus, piriform cortex, olfactory bulb, projections, retrograde tracing, retrograde labeling, HiRet, CAV2.

Chapter 1

Introduction



Introduction.

The serotonin system is considered one of the most complex neuromodulatory systems in the central nervous system (CNS) and is one of the most frequently targeted systems for pharmacological studies addressing a wide range of psychiatric dysfunctions. Anatomical studies of the mammalian serotonergic system have significantly enhanced our understanding of its anatomical and functional organization. It is widely accepted that serotonergic neurons are topographically organized with anatomically distinct groups of subpopulations receiving synaptic input from specific brain structures. The projection patterns of these subpopulations are also known to be highly organized suggesting precision in both control and projection output to a number of forebrain and brainstem circuits, which may have important implications for physiological or behavioral functions of the system[13]. This project aims to address serotonergic subpopulation projection patterns originating in the dorsal raphe nucleus by implementing labeling techniques and optogenetic tools which will eventually lead to both anatomical and functional exploration of the system.

Serotonergic System.

Serotonin is a monoamine neurotransmitter derived from the amino acid tryptophan and synthesized by a tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC) containing metabolic pathway **(Figure 1)**. Also known as 5-Hydroxytryptamine (5-HT), it is found in both the Gastro Intestinal (GI) tract and Central Nervous System (CNS) of mammals, as well as blood platelets where 5-HT is carried in large concentrations, but synthesized in small amounts and used to assist in homeostatic regulation[23]. It has been shown that serotonin is also synthesized in the placenta through a direct placental metabolic pathway that modulates the fetal brain during development[8]. However, approximately ten percent of all serotonin is produced in the CNS where it is utilized as a neurotransmitter and neuromodulator for a variety of behavioral modifications including sensory, motor and emotional responses to external stimuli[22].

The serotonergic system is both one of the evolutionarily oldest and most complex systems in the human brain with neurological pathways projecting to nearly all brain regions. Structurally, this complexity can be attributed in part to the seven different types and seventeen different subtypes of receptors that are activated by 5-HT and allow for modulation of the

system. Some of the most commonly studied of these include 5-HT1A, 5-HT1B, 5HT2A and C, and other pre- and postsynaptic 5-HT receptors[14] **(Figure 1)**. With the exception of the 5-HT3 receptor, which is a ligand gated ionotropic receptor, all others are G protein-coupled receptors (GPCRs) that are divided into four classes depending on the specific functional role in molecular signaling. Structurally, GPCRs cross the membrane seven times and are coupled to G-proteins that are intracellularly released with the binding of 5-HT to the extracellular binding sites[61] **(Figure 2)**. This release of the G-protein activates diverse signaling cascades depending on the specific 5-HT to 5-HT receptor interaction.

Pharmacological studies have helped to localize these receptors to nearly all brain regions, some spanning many regions, others more sparsely localized. In the cortical brain areas, there are at least 6 different sub-types (5-HT1A/B/E, 2A, 5A, and 6) that have been described, many of which are correlated to clinical indications related to mood and affect (e.g.. anxiety and depression)[29, 42]. However, some of these receptors are also found in other regions including the Hippocampus, Raphe Nuclei and Basal Ganglia **(Table 1)**. In addition, some receptors are localized to a single specific brain region, such as 5-HT1D found solely in basal ganglia neurons, or 5HT2A found in isocortical regions. Additional brain regions that contain 5HT receptors include striatum, hypothalamus, thalamus, cerebellum and amygdala. Though there is some variability among studies, it is generally concluded that lower levels of 5-HT and receptors are found in the cerebellum and cerebral cortex; higher levels are found in the substantia nigra, striatum and hypothalamus; while the raphe nuclei contain the highest levels of 5-HT and receptors of all brain regions[3, 4].

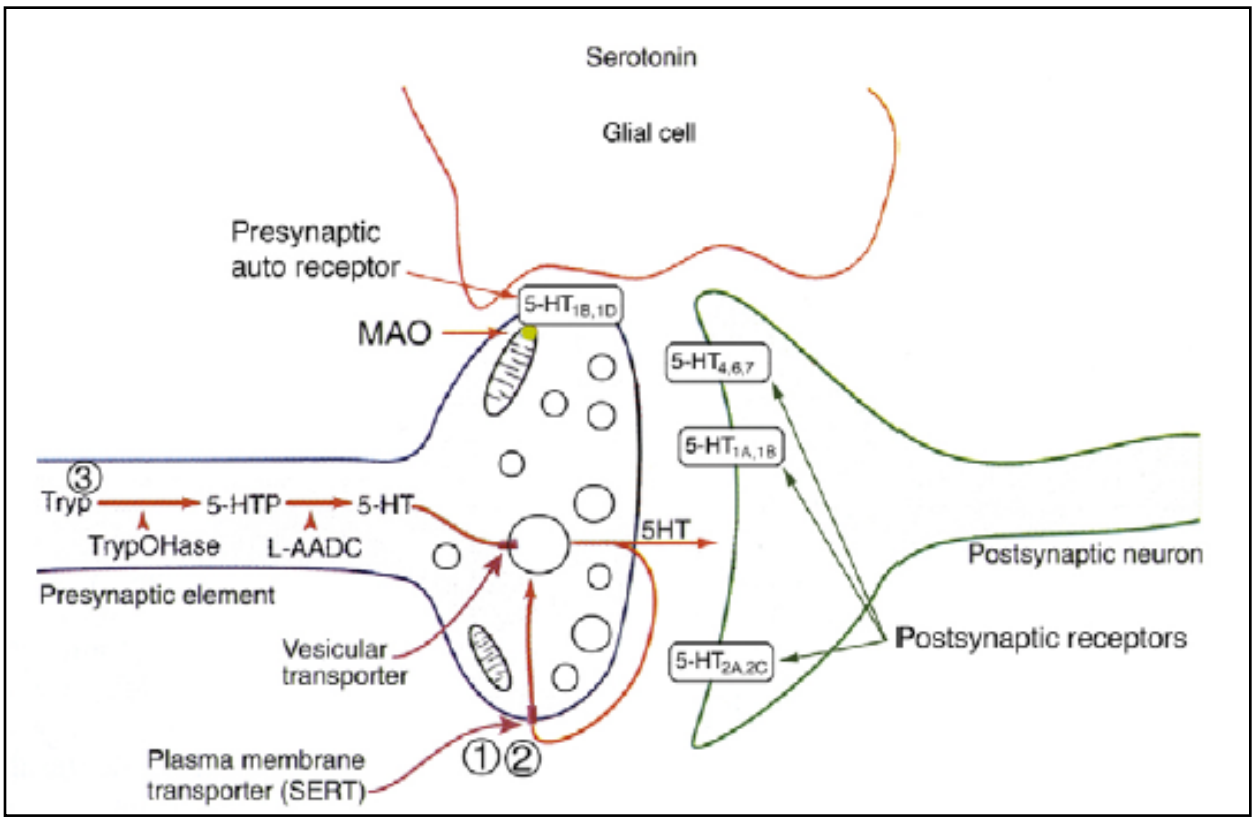


Figure 1. Schematic 5-HT (Serotonin) neuron. Tryp (tryptophan) is hydroxylated by TrypOHase (Tryptophan Hydroxylase) to form the amino acid 5-HTP (5-hydroxytryptophan). 5-HTP is converted to 5-HT through decarboxilation by L-AADC (L-amino acid decarboxylase)**.** Once packaged into vesicles, it is released into the synaptic cleft and taken up post-synaptically by 5-HT1A, 5-HT1B, 5-HT1D, 5-HT2A,C, and other postsynaptic 5-HT receptors, or pre-synaptically where it is either broken down by MAO (monoamine oxidase) or transported from the synaptic space into the pre-synaptic neuron by a plasma membrane protein, SERT (serotonin transporter), where it is then re-packaged into vesicles for future release[13, 14].

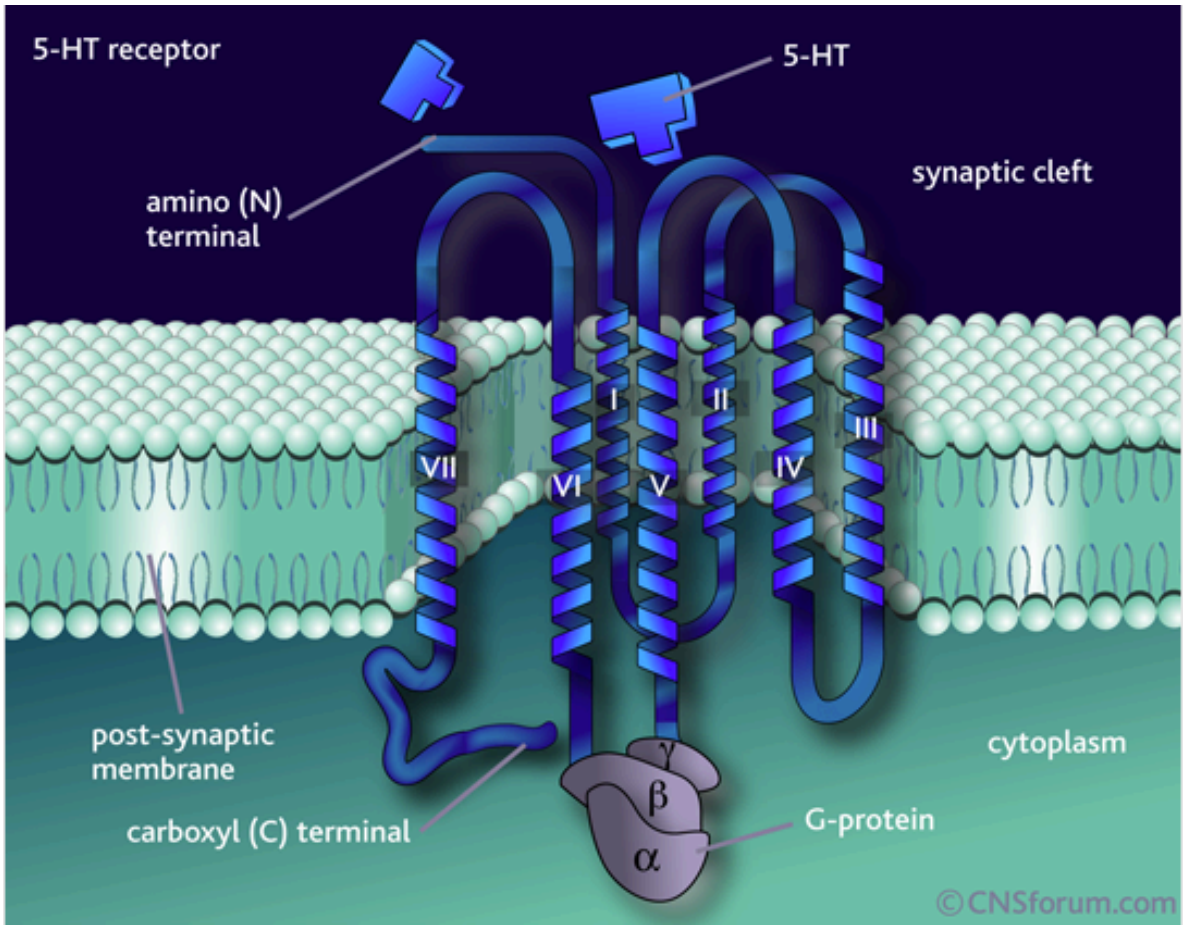


Figure 2. Illustration of 5-HT G protein-coupled receptor. The 5-HT1, 2, 4, 5, 6 and 7 receptors belong to the G-protein coupled superfamily. They are membrane receptors that have seven transmembrane spanning a-helices. 5-HT binding to the 'binding groove' on the extracellular portion of the receptor activates the G-proteins, which initiate secondary messenger signaling pathways. The downstream effect is either inhibitory or stimulatory depending on the type of G-protein linked to the receptor – 5-HT1 receptors are linked to inhibitory G-proteins, whereas 5-HT2, 4, 6 and 7 are linked to stimulatory G-proteins [60, 35].

Receptor Subtype	Brain Regions	Clinical Implications
5-HT_{1A}	Cortex, Hippocampus, DRN	Affective Control
5-HT_{1B}	Basal Ganglia, Cortical Regions	Pain, Affective Control
$5HT_{1D}$	Basal Ganglia	Pain
$5HT_{1E}$	Cortex, Striatum, Hypothalamus	Unknown
$5HT_{1F}$	Hippocampus, Cortex	Pain
$5HT_{2A}$	Isocortical Regions	Affective Control
$5HT_{2B}$	Cerebellum, Lateral Septum, Hypothalamus, Amygdala	Unknown
$5HT_{2C}$	Choroid Plexus, Basal Ganglia, Hippocampus, Hypothalamus	Feeding; Affective Control
$5HT_{3}$	Amygdala, Hippocampus	Feeding, Digestive Control
$5HT_{4}$	Basal Ganglia, Hippocampus	Affective Control; Learning and Memory
$5HT_{5A}$	Cortex, Hippocampus, Cerebellum	Unknown
$5HT_{6}$	Striatum, Cortical Regions	Learning and Memory
$5HT_{7}$	Thalamus, Hypothalamus, Hippocampus	Affective Control

Table 1. Localization of 5-HT Receptors in the CNS. General list of localization and clinical implications for 5-HT Receptors in the CNS. DRN (Dorsal Raphe Nucleus)[29, 42].

Dorsal Raphe Nucleus.

Many of the signaling cascades triggered by the release of 5-HT and its subsequent binding to 5-HT receptors originate in the Dorsal Raphe Nucleus (DRN). The Dorsal Raphe Nucleus contains a group of heterogeneous neurons located in the midbrain and Pons region of the brain. The earliest serotonergic studies utilized histofluorescence techniques revealing midline DRN localization of 5-HT neurons[17], and later staining analysis techniques were improved with the implementation of immunohistochemistry using antibodies targeting either tryptophan hydroxylase (TPH)[32], serotonin (5-HT)[64], or serotonin transporter (SERT)[76]. Additionally, mRNA encoding SERT was found in abundance in specific sub regions of the DRN that aligned with the distribution patterns associated with TPH in prior immunocytochemical studies[5]. The organization of the region became defined such that neurons located more caudally project to forebrain regions including the septum, hippocampus, and entorhinal cortex, while the more rostral neurons project to specific forebrain sites including the caudate putamen, substantia nigra, and virtually all neocortical regions[57, 64, 39].

Being the major source of serotonin to the forebrain, the DRN is only one of a number of raphe nuclei. The earliest histochemical studies were performed in rats and were the first to anatomically divide the Raphe nuclei into clusters each containing afferent and efferent projections that are highly organized[22]; this distribution of 5-HT neurons was divided into nine groups of cell clusters spread throughout the entire Raphe brain region described as B1-B9 **(Figure 4)**[17].

The caudal group consists of three nuclei spanning the area from the caudal metencephalon to the intersection of the pyramidal tract. The first of these is the most rostral of this group, the raphe magnus nucleus (RMg) containing the B3 group of 5-HT clusters. The second is the raphe obscurus nucleus (ROb) containing the B2 group, and the third is the raphe pallidus nucleus (RPa) consisting of the B1 subgroup[28] **(Figure 4)**. The rostral group consists of three nuclei spanning the area from the mesencephalon to the mid pons. The first of these is the Caudal linear nucleus (CLi) located midline in the caudal mesencephalon and containing the B8 group. The second rostral nuclei is the Median Raphe Nucleus (MRN) containing the second largest collection of 5-HT neurons in the brain. It is divided into the MRN containing the B8 and

B5 subgroup, and the pontomesencephalic reticular formation containing the B9 subgroup. The DRN is the final of these nuclei that account for approximately one third of all serotonergic neurons in the brain and contains the groups B6 (Caudal) and B7 (rostral). The DRN is further subdivided into regions **(Figure 5)** namely the dorsal division of the dorsal raphe (DRD), ventrolateral division of the dorsal raphe (DRVL), ventral division of the dorsal raphe (DRV), and intermediate division of the dorsal raphe (DRI). The DRI consists entirely of serotonergic neurons, while the DRV (80%), DRD and DRVL (60%) contain 5-HT in fewer proportions[28].

For many years, this region was thought to be solely serotonergic in property. However, other types of neurotransmitters were discovered including GABA, glutamate, dopamine and other peptide transmitters each containing distinct morphologies, projections and neurochemical characteristics, which are released or sometimes co-released along with 5-HT by the neurons in this region **(Figure 6)**[48, 28]. The first experiments were conducted using histofluorescence techniques and later antibodies were developed against tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DβH) to stain and localize dopamine cell bodies in this region[54]. These studies revealed dopaminergic localization in ventromedial areas with targets to the nucleus accumbens, lateral septum and medial prefrontal cortex (MPF)[48]. Other similar experiments used antibodies against γ-aminobutyric acid decarboxylase (GAD) and GABA-transaminase (GABA-T) to localize Gabaergic cell bodies[52, 53]. Gabaergic neurons in the DRN synapse with serotonergic neurons[72], which remain the major component of nuclei in this region controlling the release of 5-HT to the neocortex[32].

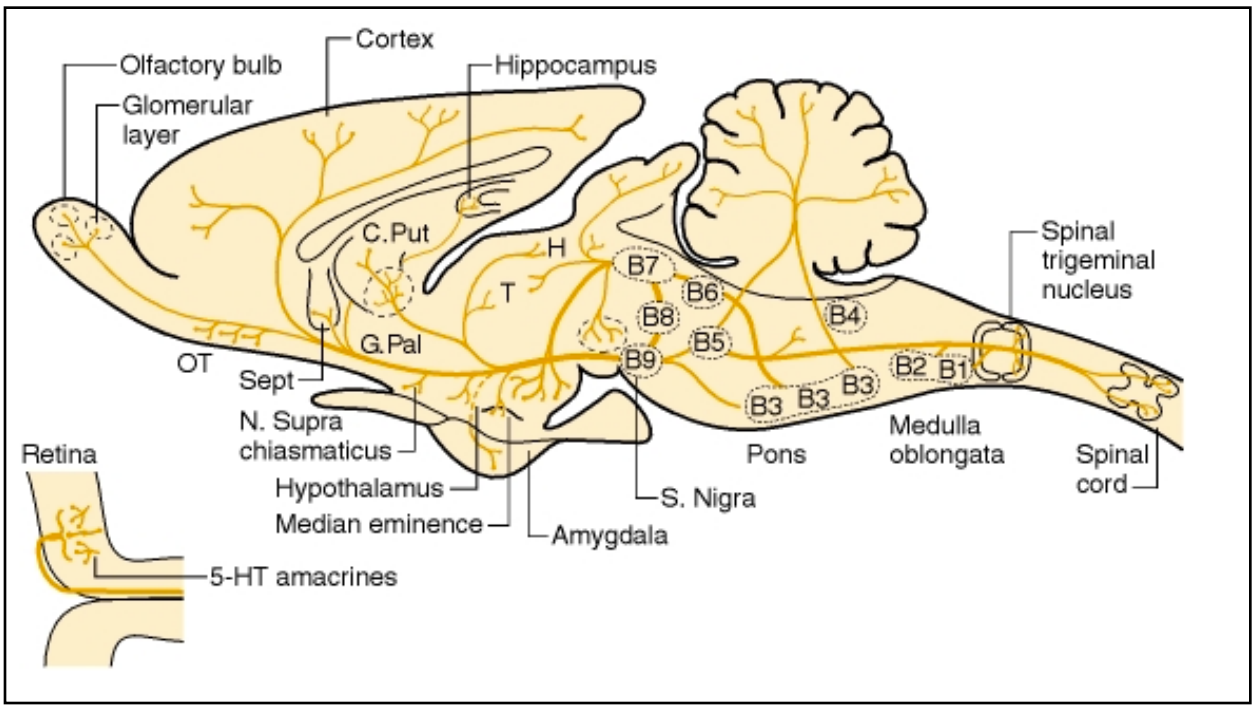


Figure 3. Schematic drawing depicting the location of the serotonergic cell body groups. Sagittal section of the rat central nervous system and their major projections. OT (olfactory tuberculum), Sept (septum), C. Put (nucleus caudate-putamen), G. Pal (globus pallidus) T (thalamus), H (habenula) S. Nigra (substantia nigra)[12, 62].

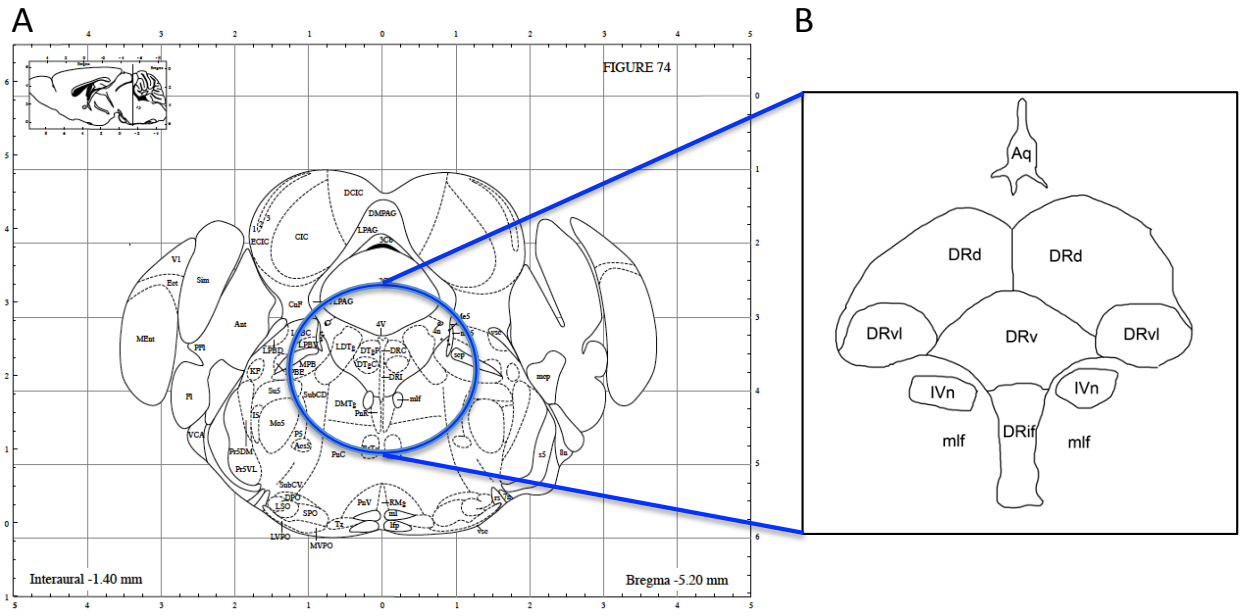


Figure 4. An Illustration of the DRN subdivisions. (a) Coronal section from *The Mouse Brain Atlas*. Blue circle localizing the Raphe with (b) a zoomed illustration depicting the DRN regions. Aq (Cerebral Aqueduct), DRd (dorsal region of the dorsal raphe), DRv (ventral dorsal raphe), DRvl (ventrolateral dorsal raphe), lVn (trochlear nucleus), DRif (interfascicular sub nucleus)[56].

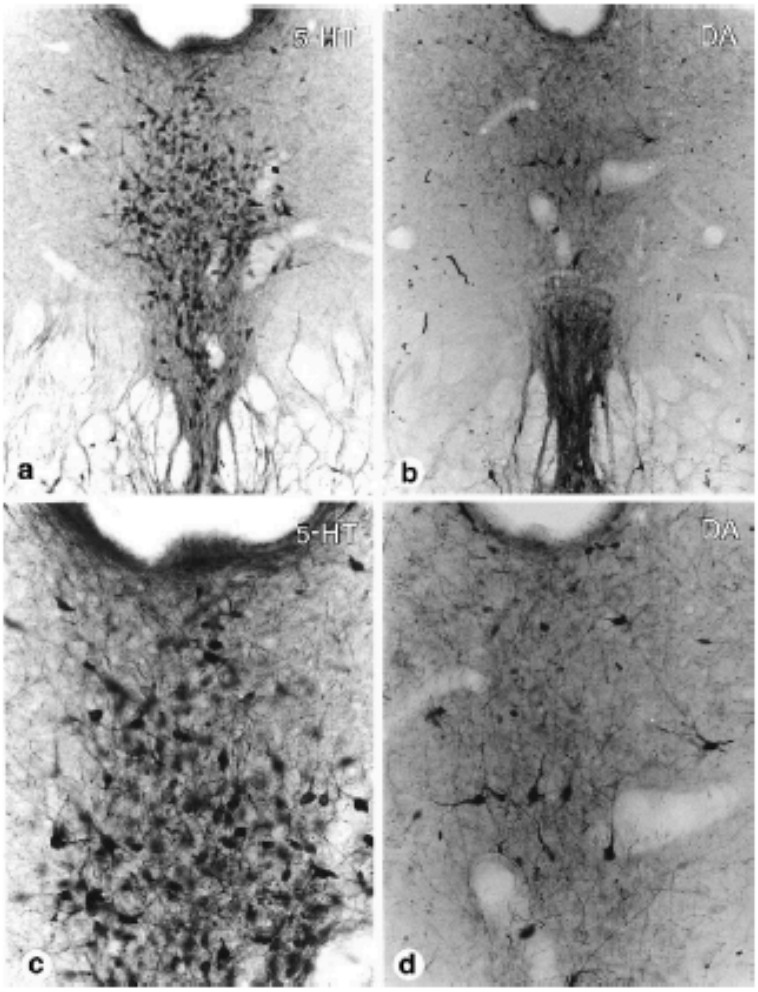


Figure 5. Immunohistochemical staining in DRN. Two of the DRN neurotransmitters, (a) serotonin and (b) dopamine visualized with DAB-immunohistochemistry in coronal rat DRN sections. Details of (a) and (b) are seen in (c) and (d), respectively[48].

Anatomical and Functional Topography

Anterograde and retrograde tracing tools have widely been used in mapping what we already know of the organization of 5-HT projections in the DRN. It is accepted that the DRN is by definition a single nucleus, yet it contains multiple subdivisions that comprise smaller groups of serotonergic neurons with differing characteristics and projections that can been seen throughout the entire brain **(Figure 6)**. The patterns of these neurons are highly organized suggesting unique functional characteristics that could be associated with the structural mapping of the system. For example, serotonergic neurons located more caudally project selectively to specific forebrain sites (e.g., the septum, hippocampus, and entorhinal cortex), while serotonergic neurons located more rostrally project to other forebrain sites (e.g., the caudate putamen, substantia nigra, and almost all neocortical regions). This means that some functional specificity can be attributed to the structural organization within these subpopulation regions of the DRN [64].

The earliest experiments exploring structural organization used retrograde labeling tracers injected into different, but functionally related sites in the forebrain[22]. Retrograde tracers were injected in pairs (caudate putamen paired separately with the substantia nigra, amygdala, hippocampus, and locus ceoeruleus (LC); amygdala with hippocampus, LC; and hippocampus with LC). Results were similar to subsequent findings; the rostral part of the DRN sends collateral projections to the caudate putamen, while caudal parts send projections to regions of the limbic system such that these collateral serotonergic projections could allow modulation of specific neural circuits implicated in affective functioning and control including conditioned fear and stress response[40]. This sets up a hypothesis that different regions of the DRN could have specific and organized functional characteristic based on projection targets of that region.

Additionally, the dorsal part of the DRN (DRD) projects to the amygdala, hypothalamus, nucleus accumbens and prefrontal cortex; the median raphe nucleus (MRN) to septohippocampal system and to cortical regions involved in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis[69]; the ventral part of the DRN (DRV) to the caudate putiman, sensorimotor cortex, frontal cortex and motor cortex; the ventral lateral (DRVL) to the sympathomotor control system, subcorticol structures and DRV; and the lateral vestibular nucleus to the dorsal/ventral hippocampus, medial septum and cortical regions[22].

Specific bundles of 5-HT subpopulations are found within subregions. It is clear that the organization at present remains complex and broad, yet it serves as a general map of subregion organization that might be used as a starting point for further experimentation of DRN labeling. The literature also suggests variable definitions to distinguish these subgroups within the DRN, with some definitions of caudal regions being the same as others' definitions of rostral [59, 37, 41]. The questions remain whether we can further understand the organization of these subregions, whether a universal acceptance of subregion divisions can be defined and whether these specific subpopulations can be associated with sensory input and behavioral output patterns. The more the anatomical specificity of these projections and their organizational patterns with the DRN is elucidated, the more this information will be utilized to control and manipulate specific serotonergic brain circuits, which will lead to a further understanding of the multifaceted functions of this system.

5-HT and Behavior

Perhaps the view supporting functional heterogeneity of the DRN is more realistic and accepted among researchers compared to a single function hypothesis. 5-HT has been shown to play a key role in the regulation of physiological, cognitive and affective processing including sleep, mood, feeding, thermal regulation, and memory; therefore dysfunction or disruption of the system is linked to a number of disorders ranging in scope from physiological to psychiatric classification. Additionally, there remains much confusion about the role of 5-HT, which is not surprising considering the vast complexity of the system, the broad anatomical connectivity of its projections and the paradoxical nature as it relates to behavior.

Affective Control

Serotonin is one of the most important targets for pharmacological studies addressing mood disorders (ie., depression, anxiety, panic attacks and aggressive behavior)[19]. The earliest studies to link 5-HT to mood measured serotonin levels in the cerebral spinal fluid of humans diagnosed with major depressive disorder. These patients showed low-levels of serotonin metabolites, as well as decreased levels of available L-tryptophan in the blood plasma[15]. More recently, serotonin depleted animal models are being used to study the effects of serotonin on behavior. *Tph2*-deficient (*Tph2*−/−) mice have been used to evaluate the impact of serotonin depletion during a number of behavioral tasks designed to evaluate depressive-like behaviors in animals. *Tph2*−/− mice exhibited increased depression-like behavior in the forced swim test, showed decreased anxiety-like behavior in the elevated plus maze task, and exhibited aggressive-like behavior during the resident-intruder paradigm[51]. These results support the hypothesis that low-serotonin levels are linked to depressive-like symptoms, as well as aggressive-like behavior.

However, the paradox of 5-HT emerges in that 5-HT is implicated in both depression and anxiety, as well as panic disorder and aggression, such that the system produces contradictory responses to 5-HT dysfunction[13]. This is also seen within the framework of current pharmacological treatments most widely used to reduce anxiety in psychiatric conditions, such as selective serotonin reuptake inhibitors (SSRIs) by increasing 5-HT transmission[27], and benzodiazepines by reducing 5-HT transmission[13]. It is unclear how one system has the ability,

both on its own and in tandem with other neuromodulatory systems, to control and regulate such a wide range of behaviors that are at times contradictory in function. The brain circuitry involved is also not well-defined, but much of the research points to the limbic brain areas as possible targets for affective control[67]. The limbic brain circuitry includes a number of neural pathways such as corticolimbic, dorsal raphe, hippocampal, amygdalar, striatal and mesolimbic dopamine circuits. The limbic circuit is a target of interest not only in affective control, but in memory, learning, motivation and olfaction, and is known for its control of the 'fight or flight' mechanism in mammels. Serotonin projecting neurons from the DRN target the limbic brain areas and are often a focus in serotonin research as it relates to emotional control processes, affective dysfunction and treatments for a wide range of affective disorders **(Figure 7)**[73].

5-HT can also mediate and interact with other neuromodulators including dopamine, GABA and glutamate in order to exert its role in affective functioning[19]. For example, corticotropin-releasing factor (CRF) has been shown to modulate serotonergic neuron activity through its interaction with GABAergic neurons in the DRN, and that CRF receptors upregulate and downregulate GABAergic neurons, which inhibit 5-HT neurons. Additionally, Galanin, a neuropeptide encoded by the GAL gene, has been shown to have antidepressant-like effects through the upregulation of serotonergic transmission via galanin receptors on 5-HT DRN neurons[48]. Additionally, other theories have been proposed that suggest that rather then exerting direct affective control, 5-HT could either be modulating representational and functional responses to aversive events such that aversive processing can change in response to the level of serotonin available at it's receptors, or alternatively 5-HT's affective outcomes could be a result of behavioral inhibition[16].

Behavioral Inhibition

Early behavioral studies suggested the involvement of serotonin as a neuromodulator in withholding responses particularly when linked to aversive stimuli, such that lowered serotonin transmission produced a decrease in withholding punished responses[63]. More recently, neuroscientists are trying to separate these two areas, behavioral inhibition and aversive response, by either suggesting a distinct role for serotonin in aversive processing, or proposing a

more general role for serotonin in behavioral inhibition[16]. It has been shown that aversive events such as inescapable shock activate the release of 5-HT from neurons[66], but depleted serotonin levels are correlated with an increased behavioral response to aversive cues and contexts[63], and temporarily lowering serotonin in humans seems to enhance aversive processing in cognitive tasks[13]. Paradoxically, these studies suggest that if 5-HT is involved in aversive processing, then depleting it should have positive affective consequences as opposed to the many studies linking low 5-HT levels to depressive-like symptoms[19], so these contradictory findings are difficult to understand.

More recently, acute tryptophan depletion has been used to study the effects of low serotonin levels to try understand and separate the hypotheses that link serotonin to motor response inhibition, punishment-induced inhibition, or affective sensitivity to aversive outcomes. In human studies, subjects given placebo were slower to respond under punishment conditions compared to reward conditions, while tryptophan depletion abolished this punishment inhibition response and did not have any affect on motor response or the ability to adjust response to punishment conditions. Additionally, the level of reduction in punishment-induced inhibition depended on the degree of reduced plasma tryptophan levels. These findings support the current hypotheses linking serotonin to aversive processing and aversive outcome prediction. as opposed to motor response inhibition[16].

However, it has also been argued that the behavioral inhibition that results from aversive outcome prediction is not sufficient to explain some forms of impulsive behavior in animals. Recent microdialysis and unit recording studies examined the 5-HT neuronal activity in behaving animals and found an increase in 5-HT release when rats performed a task that required waiting for delayed reward[49, 50]. Other results have confirmed an increase in serotonergic neuronal firing that facilitates waiting behavior when linked to a future reward. This suggests that the inhibitory effect of 5-HT is not solely linked to aversive prediction. The medial prefrontal cortex (mPFC) and Orbitofrontal (OFC), both of which have reciprocal connections with the DRN, are brain regions involved in waiting tasks. They have been shown to sustain increased activity during waiting for delayed rewards, however the role of the OFC does not seem to contribute directly to response inhibition, but rather to signal expected outcomes to other brain regions[49, 50]. The OFC

circuit has both efferent and afferent connectivity to both limbic and motor control regions as well causing an ongoing debate as to whether the behavioural inhibition associated with raised levels of 5-HT is best explained in motivational or motor terms[13].

Sensorimotor Gating

Although there is little information regarding the mechanistic circuits for serotonin's role in motor inhibition under non-punishment conditions in humans and rodents[11, 20], there is evidence that points to both motor connectivity of serotonergic pathways and sensorimotor modulation in various sensorimotor modalities. The earliest motor studies used serotonergic pharmacology to study effects of 5-HT perturbations on motor function in rats resulting in symptoms of hyperactivity and tremors, or an increased amplitude motor response in hind limb function in cats[65, 6]. It has also been suggested that 5-HT modulates respiratory activity, as well as feeding, avoidance and escape behavior[19].

Anatomically, there is evidence supporting motor influence of 5-HT projections on behavior. For example, the brain stem and spinal cord α-motoneurons receive serotonergic inputs in the ventral horn, the motor nucleus of the trigeminal, and the facial motor nucleus[31]. In the spinal cord, serotonergic inputs innervate motoneurons projecting to axial rather than distal structures, while inputs to the brain stem are directed toward large projections of jaw, face and neck muscles. 5-HT neurons also innervate secondary structures such as substantia nigra, globus pallidus and habenula, all structure associated with motor and/or sensory function. Jacobs and Fornal[31] propose an interesting hypothesis suggesting when motor activity is initiated, sensory information processing is suppressed, so that 5-HT neurons play a continuous inhibitory role in sensory transmission.

In rodents, smell is behaviorally the most relevant sensory modality. In the olfactory bulb, which is the first stage of olfactory information processing, serotonergic fibers that originate in the brainstem raphe nuclei preferentially project to the glomerular layer of the olfactory bulb[46], and according to one study, 5-HT released from the raphe nuclei inhibits glomurular activity[58]. Raphe neurons are known to modulate their activity in longer time scales with different states of wakefulness and arousal, and in shorter time scales in response to salient

events. These findings support the hypothesis that an increase in firing of raphe neurons, which may occur during periods of higher sniffing rate, leads to a decrease in the sensory evoked activity in the olfactory bulb. It is therefore hypothesized that the overall gain of olfactory input is reduced by 5-HT to keep the postsynaptic responses in a normal dynamic range[58] to prevent them from being saturated supporting prior theories relating 5-HT to sensorimotor gating. Additionally, the second stage of olfactory processing, Piriform Cortex (PC), is also densely innervated by 5-HT fibers originating in the DRN. However, the functional role of 5-HT in the PC is poorly understood.

Many of the functions effected by serotonin modulation are directly linked to the structural projections previously mapped within the system. There are some common characteristics shared by affective control and inhibitory and sensorimotor processes associated with these 5-HT pathways and disentangling the complexity of 5-HT system on both an anatomical and functional level will help to understand these characteristics more clearly.

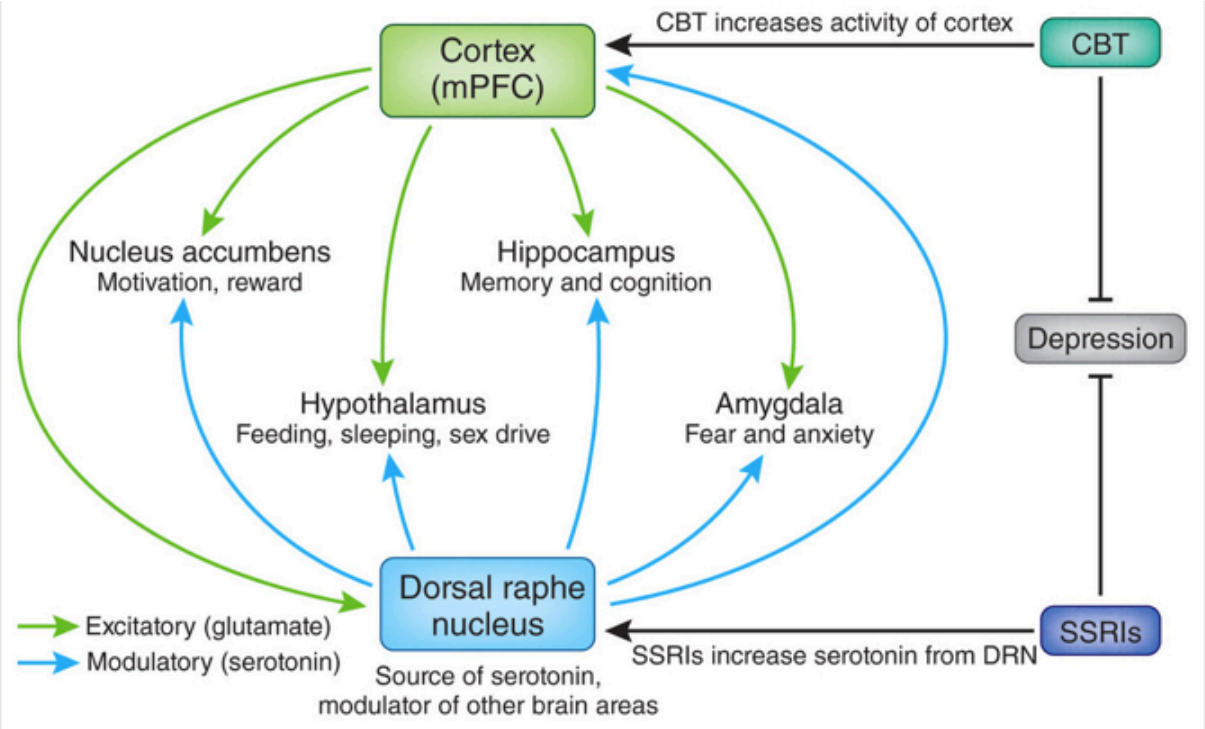


Figure 6. 5-HT Projections from the DRN to limbic brain areas. Serotonin pathways in blue project from the DRN to nucleus accumbens, hypothalamus, hippocampus and amygdala, while glutamate pathways in green project from the medial Prefrontal Cortex (mPFC) to these same regions. Treatments for depression exert effects on either the DRN or mPFC; SSRIs to the serotonin pathway, and Cognitive Behavioral Therapy to the glutamate pathway[73].

Retrograde Labeling Methods

Retrograde labeling tools are used to assist neuroscientists in understanding how neurons generate behavior by mapping circuitry within the CNS. The functions of the brain are based on the activity patterns of large populations of interconnected neurons that form neural circuits. Each type of retrograde tracer has its own set of characteristics that determine the scope of problems it can address. Retrograde tracers are injected into the projection site, taken up by axons and carried to cell bodies where they are expressed. There are both advantages and limitations to using any of the diverse range of retrograde tracers available.

Latex Microspheres.

Latex microspheres have commonly been used to study structural anatomy within the CNS. They are 0.02mm polymeric particles that have been suspended in latex and tagged either with green (fluorescein) or red (rhodamine) fluorescent markers. Once injected into the region of interest, they take a relatively short time to express within cells bodies, are known for their ease-of-use, high transport efficiency, distinctive cell labeling, the ability to produce well-defined injection sites and can be imaged using fluorescence microscopy[1] **(Figure 7)**. They have successfully been used for decades to map anatomical projections in the CNS. The earliest studies looked at retrograde transport in cat visual cortex slices and revealed significant differences in patterns of intrinsic axonal and dendritic arborization[34]. Today, they are widely used as a general anatomical marker in many experimental protocols. Latex microspheres provide a means of visualizing pathways of interest, but are limited to structural anatomy; they cannot be used for studying functional anatomy or behavior.

Viruses.

Retrograde virus methods can reveal mono- or multi-synaptic pathways and can identify connections to and from particular cell types to guide functional studies that have higher components of complexity. Viruses are injected into the target region and transported retrogradely to cell bodies where transgene expression takes place **(Figure 8)**. There are different types of viruses and techniques that can be used each with advantages and limitations. Some advantages are the ability to traverse multi-synaptic pathways, the ability of viral replication and

high specificity to synaptically connected partners[38]. However, some limitations include difficulty in cell infection, difficulty in retrogradely transporting large constructs from the injection site especially over longer distances, and a lack of cell-type specificity.

A Cre recombinase-dependent system can be implemented to help solve the limitations of cell type specificity and construct size[2] **(Figure 9)**. There are two strategies that can be utilized for visualizing and eventually controlling specific cell types. One is to inject a retrograde virus containing a vector with a specific promoter, such as Pet1 for serotonin neurons, into a target region in a transgenic mouse containing a stop codon between loxp recombinase sites that is followed by genes of interest (these genes may be for example Channelrhodopsin-2 for controlling neuronal activity or enhanced yellow fluorescence protein for visualization). When the virus interacts with the loxp site, the stop is excised only in cells expressing genes under the specific promoter. The second strategy is to inject a virus containing genes of interest and loxp-sites into a transgenic mouse in which Cre is expressed in only a subset of neurons (such as the SERT-Cre mouse for serotonin specific neurons), so that only cells expressing Cre will express the genes of interest following virus injection. These techniques are useful both for visualizing projection pathways, and for future use with Optogenetic strategies.

Rabies Virus.

Rabies Virus (RV) is useful in its ability to spread transynaptically solely in the retrograde direction, and infected cells stay viable for weeks. It also has the ability to amplify from one single particle making it highly efficient[74]. The single biggest drawback in using RV is that it is a negative strand RNA virus, which makes it difficult to develop novel variants necessary for use with genetic tools and transgenic mouse lines. Although recovery of new RV variants is difficult, it can be accomplished; recent production of a new SADΔG variant and an efficient method for recovery and amplification[55] was established, so that variants that encode a multitude of tools were developed including fluorescence proteins, Channel Rhodopsin-2 (ChR2), and flippase (FLP) recombinase. However, these are difficult to acquire, and RV is highly toxic in property, which makes it a less desirable candidate when other options are available.

Adeno-associated Virus.

Adeno-associated Virus (AAV) is a single stranded virus for which nine serotypes have been described with humans as the primary host. It can be used for retrograde tracing and is an ideal candidate in that it lacks pathogenesis. It can infect both dividing and non-dividing cells, has the ability to persist for long periods. It has limited retrograde transport ability, so injecting an AAV into a target region may not result in efficient transport along axons. **(Figure 9b) (Table 2)**.

Canine Adenovirus Type 2

A high level of retrograde transport and preferential transduction of neurons make Canine Adenovirus Type 2 (CAV2) vectors ideal tools to study the pathophysiology of neuronal networks, as well as map complex circuits *in vivo*. CAV-2 vectors have been used in conjunction with Cre recombinase system and has been shown to express more effectively then AAV[25, 26]. Because CAV-2 is more difficult to clone compared to AAVs achieving cell-type specificity is more challenging **(Table 2)**.

Highly Efficient Retrograde Gene Transfer.

The highly efficient retrograde gene transfer (HiRet) is a pseudotype of the HIV-1 lentiviral vector with FuG-B, which is composed of the extracellular and transmembrane domains of RV-G and the cytoplasmic domain of VSV-G, which will introduce a transgene into neurons that innervate a particular brain region at the vector injection site and confer a fundamental tool for genetically manipulating specific neural pathways[33]. Compared to other viruses, HiRet is easier to clone. This offers an option for injecting HiRet with a serotonin-specific promotor (HiRet-Pet1-Cre) into the target region of ChR2 transgenic mouse (Rosa26-LSL-ChR2H134R-EYFP), which would allow for highly efficient retrograde transport and serotonin specificity. However, since this is a novel approach, it will take time to develop and optimize **(Figure 9a) (Table 2)**.

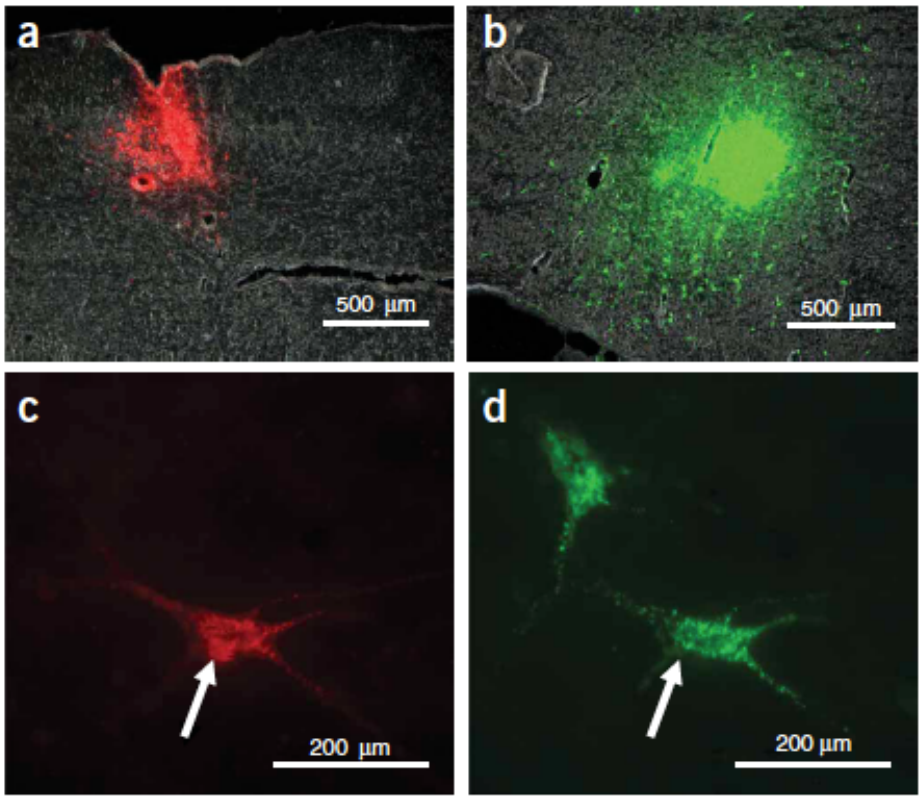


Figure 7. Typical injection sites using red and green latex microsphere. (a, b) Caudal brainstem injection in rat using red (a) and green (b); (c, d) Lumbar spinal cord using red (c) and green (d)[1].

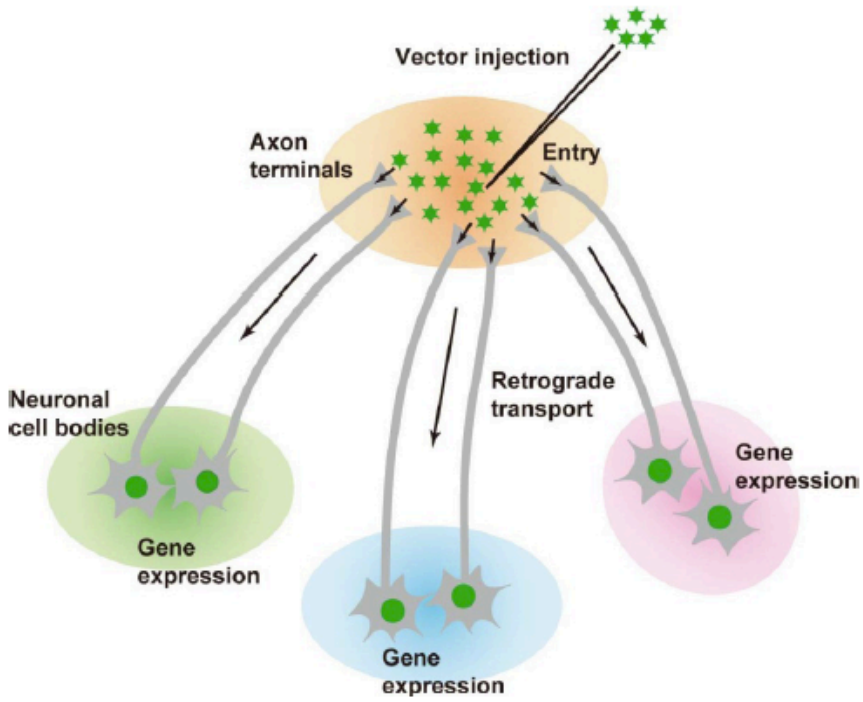


Figure 8. Schematic of virus transport and expression. Viral vectors are injected into projection site of interest, then picked up by nerve terminals and retrogradely transported along axons until they reach cell bodies where transgene expression takes place[33].

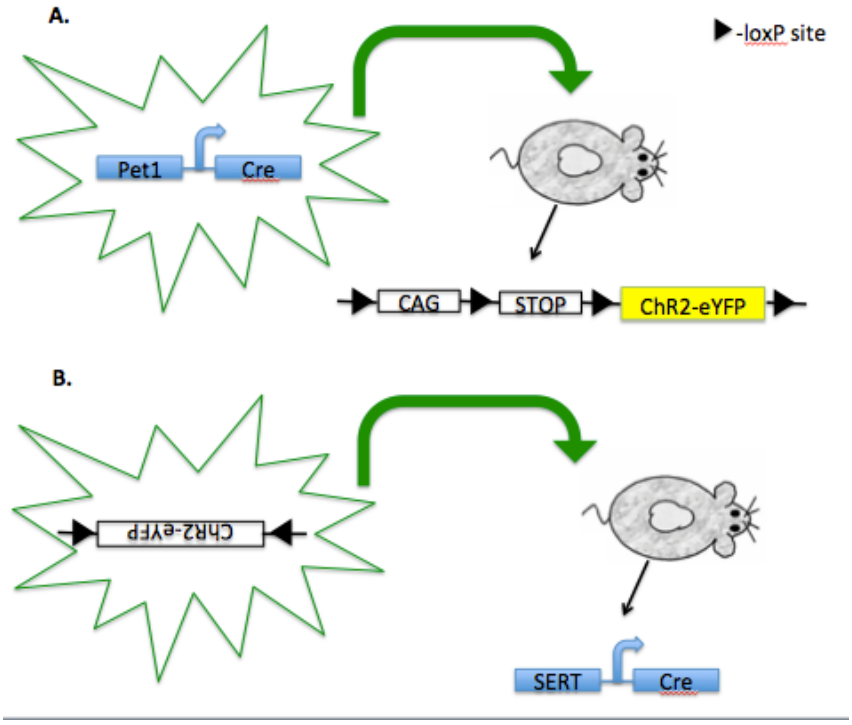


Figure 9. Cre-lox recombinant system. Design and Characterization for (A) Virus containing Pet1-Cre for serotonin specificity injected into transgenic mouse line (Rosa26-LSL-ChR2H134R-EYFP). When Cre interacts with the loxP sites it will excise the stop and expression of ChR2-eYFp will be seen in serotonin cells. (B) Virus containing flexed ChR2-eYFP is injected into a SERT-Cre transgenic mouse. When Cre, which is expressed only in serotonin cells, interacts with loxP sites, the genes are inverted and ChR2-eYFP is expressed in serotonin cells.

Virus	Construct/Strategy	Pros	Cons	Reference
RV	Unavailable	Good retrograde transport, good viability	Toxic, difficult to develop new variants	Osakada et al., 2011
AAV	Double floxed ChR2 injected into a SERT-Cre mouse	Good axonal and cell type specificity	Difficult to clone, limited retrograde transport	Betley and Sternson, 2011
CAV2	Double injection: General promoter Cre in target region + double floxed ChR2 in DRN	Good retrograde transport	No cell-type specificity	Hnasko et al., 2006
HiRet	HiRet-Pet1-Cre injected in target region of ChR2 transgenic mouse (ai32)	Good specificity	Unknown viability, relatively new virus	Kato et al., 2011; Kinoshita et al., 2012

Table 2. Retrograde Virus Possibilities for Proposed Study. Table depicting the advantages and limitations of different viral vectors and strategies for mapping of serotonin circuits in the CNS.

Aim of Proposed Study.

The aim of this project is to develop and/or implement labeling methods to visualize and control subpopulation projection pathways of 5-HT neurons originating in the dorsal raphe nucleus. This project will be twofold. First, retrograde labeling tools will be explored and optimized. This will include the use of retrograde latex microspheres and the development of viral strategies as tools for optogenetic control. The first stage will be to optimize the following: stereotaxic coordinates for injection sites in the regions of interest, experimental protocols (i.e., immunohistochemistry) for visualizing co-localization of cells, as well as imaging and analysis techniques. Second, will be to gain a structural understanding of the localization patterns of cells originating in the DRN and projecting to the regions of interest, and to determine whether or not these cells contain 5-HT. The regions of interest include the Piriform Cortex (PC) and Olfactory Bulb (OB) as they receive projections from the DRN.

Piriform Cortex

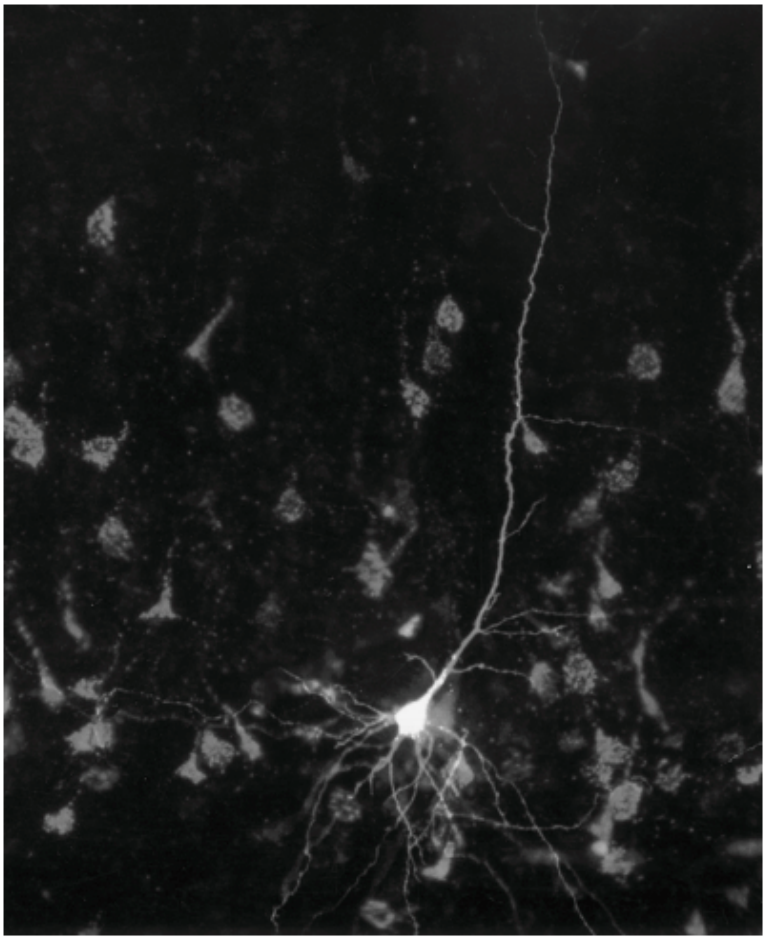
5-HT projections have been documented from the Raphe to the PC using retrograde labeling of Cholera-toxin B coupled with immunohistochemistry with specific cells labeled in the medial ventral DRN[18]. There is known to be high 5-HT innervation in this region, as well as non-serotonergic innervation from the mitral cell layer of the olfactory bulb[24], which will offer a clear landmark to visualize the integrity of the retrograde tracer and the accuracy of the injection.

Olfactory Bulb

There is some ambiguity regarding known projections to the OB from the midbrain raphe with some studies supporting a prominent role for DRN projections, while other studies argue for MRN. Importantly, it is not known whether the 5-HT neurons that project to OB are the same neurons that project to PC. This may have significant implications for the functional impact of 5-HT on the olfactory system.

Chapter 2

Materials & Methods



Materials.

Dolorox and Trivertan were purchased from Intervet Schering-Plough (Paço de Arcos, Portugal). Visidic Gel was purchased from Angelini Farmacêutica Lda (Bausch & Lomb) (Oeiras, Portugal). Isofuorene was purchased from Vetfluor Virbac de Portugal Laboratórios Lda (Sintra, Portugal). Saline was purchased from B. Braun Medical, Lda (Queluz de Baixo, Portugal). Gentimiacin, Paraformaldehyde, Sodium hydroxide, Monopotassium phosphate, Disodium phosphate, Sucrose, Tris-sodium Citrate containing Tris, Hydrochloric Acid, Triton X-100, Tris Buffered Saline containing Tris, Sodium Chloride and Potassium Chloride, 10% bovine serum albumin diluent blocking solution and Mowiol were purchased from Sigma-Aldrich (Sintra, Portugal). 5% Goat Serum was purchased from Life Technologies (Barcelona, Spain). Red Lumafluor Retrobeads™ IX and Green Lumafluor Retrobeads™ IX were purchased from Lumifluor Inc.

Antibodies.

Monoclonal Anti-Tryptophan Hydroxylase antibody produced in mouse (α-TPH) (T0678 Sigma) was purchased from Sigma Aldrich. Alexa Fluor® 488 Rabbit Anti-Mouse IgG (H+L) labeled with bright, photostable, green-fluorescent Alexa Fluor 488 dye and Alexa Fluor® 647 Goat Anti-Mouse IgG (H+L) labeled with bright, fluorescent far-red Alexa Fluor 647 dye prepared from affinity-purified antibodies that react with IgG heavy chains and all classes of immunoglobulin light chains from mouse were purchased from Life Technologies (Porto, Portugal). DAPI Nucleic Acid stain was purchased from Sigma Aldrich (Sintra, Portugal).

Animals.

8-12 week old male (250-300g) Sert-CRE or Ai32 (Rosa26-LSL-ChR2H134R-EYFP) transgenic mice were used. Animals were housed in group cages with ad libitum access to food and water. The room was maintained at 21°C, on a 12h:12h light/dark cycle (8am-8pm), with a light intensity of 323-377 lux and humidity of 30-70%. All procedures were performed in accordance with the Champalimaud Foundation Ethics Committee guidelines, and approved by the Portuguese Veterinary General Board (Direcção Geral de Veterinária, approval ID 014315).

Generation of transgenic mice.

The Slc6a4-Cre line (ET33, also called SERT-Cre)[21] was obtained from MMRC (Mutant Mouse Regional Resource Center) and were then produced by breeding +/+ and SERT-Cre/+ mice. The Ai32 line (Rosa26-LSL-ChR2H134R-EYFP)[43] was obtained from The Jackson Laboratory. The Double transgenic mice SERT-Cre/+; Rosa26-LSL-ChR2/+ were produced by breeding SERT-Cre/+ and Rosa26-LSLChR2/+ mice.

Surgeries.

Mice were initially anesthetized in an induction chamber primed with isofluorane, then mounted and stabilized on a stereotaxic frame where they were maintained in an anesthetized state using a gas mixture with oxygen (1 L/min) and isofluorane (1.5 - 2 L/min). Subcutaneous injections of Dolorox (0.1ml in 9.9ml 0.9% Saline, 0.1ml/10g) and Trivertan (0.04ml in 9.96ml 0.9% Saline, 0.1ml/10g) were administered each in 0.25-0.30ml doses prior to incision. A midline incision was made exposing the skull, and Bregma and Lambda were aligned and used as points of reference. A small hole was drilled into the skull using a dental drill at points corresponding to the injection locations listed below. Injections were made using a Picospritzer III (Intracel) fixed with a quartz micropipette (tip size 1.0 mm). Solutions were injected at a rate of approximately 0.04µl/min. The pipette was held in place for 5 minutes after injection, then immediately retracted. The head skin was then sutured and an application of Gentimiacin (0.3% in 0.9% Saline) was administered. Mice were housed individually post-surgery and sacrificed 2-5 weeks after injections as outlined below according to the type and location of retrograde tracer being injected.

Retrograde Tracers.

Latex Microspheres.

Red or Green IX (undiluted) Lumafluor Retrobeads™ (0.125µl) were injected into the Piriform Cortex (from Bregma, 2.5mm lateral; from midline, 2.2mm anterior; from dura 3.4 mm deep), the Olfactory Bulb (from Bregma, 0.75mm lateral; from midline, 4mm anterior; from dura, 0.75mm deep), Raphe (from Bregma, -4.75mm; from midline, 0mm; from dura, 3.1 at an

angle of 32°) or VTA (from Bregma, -3 mm; from midline, 0.5 mm; from dura, 4 mm) corresponding to coordinates laid out in the *The Mouse Brain in Stereotaxic Coordinates*[56]. Beads were stored at 7°C and protected from light. A post-injection survival time of 2 weeks was adhered to to allow for complete transport of beads to the Raphe, though no increase in fluorescent intensity is observed after 48 hours.

Virus Generation.

The AAV2/1.EF1a.double floxed ChR2.EYFP was purchased from the Penn Vector Core, School of Medicine, University of Pennsylvania (USA). The CAV2-Cre and CAV2-GFP viruses were a gift of Eric J. Kremer (Institut de Génétique Moléculaire de Montpellier CNRS-UMR 5535 - 1919, Route de Mende - 34293 Montpellier, France). The lentivirus DNA Hi-Ret-MSCV-GFP was a gift of Kazuko Kobayashi (Department of Molecular Genetics, Institute of Biomedical Sciences, Fukushima Medical University School of Medicine, Fukushima, Japan). The virus was produced by the CCU Vector Production Platform (Tatiana Vassilevskaia). The Hi-Ret-Pet1-Cre DNA construct was cloned by Enrica Audero of CCU by replacing MSCV-GFP with Pet1-Cre.

Tissue Preparation

The mice were anesthetized with an intraperitoneal injection of Eutasil (.025-.030), then perfused transcardially with 4% paraformaldehyde (4% PFA) in 0.1 M phosphate buffer (PB). The brains were postfixed overnight in 4% PFA solution, then immersed for 48 hours in PB containing 30% sucrose. Coronal sections (12-20 μm thick) were prepared using a cryostat, and slices were then mounted and sealed or left at room temperature for immunofluorescence staining.

Immunofluorescence Staining.

For immunostaining, heat induced unmasking was performed on Raphe sections (12-20 μm thick) followed by 3 times wash in Tris-buffered saline pH 7.5 (TBS). Slices were then blocked in a blocking solution containing 5% Goat Serum, 2% bovine serum albumin (BSA),

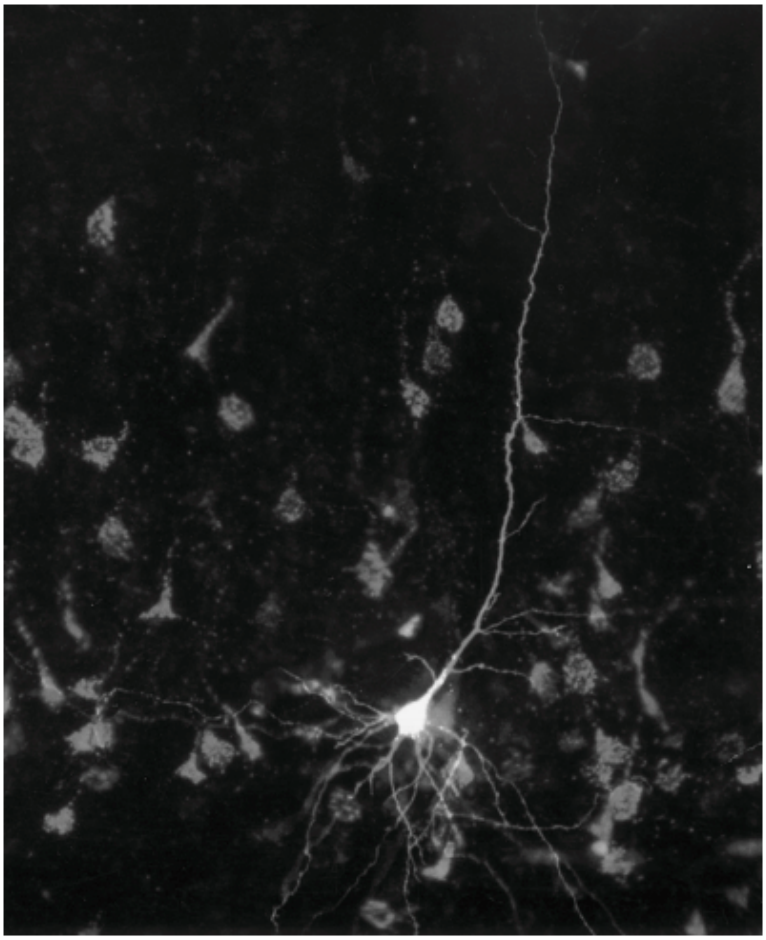
and 0.25% Triton X-100 in TBS for 1 hour followed by 3 times wash in TBS pH 7.5 and an immediate incubation in Monoclonal Anti-Tryptophan Hydroxylase antibody produced in mouse (α-TPH) diluted 1:400 in blocking solution containing 5% Goat Serum, 2%, BSA and 0.25% Triton X-100 in TBS for 1 hour at room temperature. As the secondary antibodies, Alexa Fluor® 488 Rabbit Anti-Mouse IgG (α-mouse) diluted 1:1000 in blocking solution containing 5% Goat Serum, 2%, BSA and 0.25% Triton X-100 in TBS and Alexa Fluor® 568 Goat Anti-Mouse IgG (α-mouse) diluted 1:1000 in blocking solution containing 5% Goat Serum, 2%, BSA and 0.25% Triton X-100 in TBS were used depending on the desired fluorescence. Dapi staining was used as a control for all experiments and coverslips were mounted and sealed using Mowiol.

Image Acquisition and Analysis.

Images were acquired using an upright Widefield Fluorescence Microscope equipped with a motorized stage for tiled image acquisition and stitching (Carl Zeiss Axioimager.M2) controlled with Axiovision 4.8.2 software. Each image was acquired using an EC Plan-Neofluar 20x/0.50 objective and filtersets corresponding to four different wavelengths. DAPI fluorescence was detected using a G365 excitation filter (emission peak: 365 nm), a 395 nm dichroic mirror and BP 420-470 nm emission filter. GFP fluorescence was detected using a high efficiency filterset consisting of a BP 450-490 nm excitation filter, a 495 nm dichroic mirror and a BP 500-550 nm emission filter. DsRed fluorescence was detected using a high efficiency filterset consisting of a BP 538-563 nm excitation filter, a 570 nm dichroic mirror and a BP 570-640 nm emission filter. Cy5 fluorescence was detected using a BP 625-655 nm excitation filter, a 660 nm dichroic mirror and a BP 665-715 nm emission filter. Tiled images were acquired in brightfield and fluorescence with exposure times optimized for each channel and stitched together using the AxioVision software. An in-house developed Matlab application was used to visualize merged images and identify beads in the Raphe. Cells were counted manually; beads were counted in the Raphe, and then categorized according to the positive or negative co-localization with 5-HT cells.

Chapter 3

Results and Discussion



Retrobeads.

In the first part of this study, retrograde latex microspheres (Lumifluor Retrobeads) were injected in either the Piriform Cortex or Olfactory Bulb to analyze transport to and localization in the Dorsal Raphe Nucleus. Cell counting was performed manually, and co-localization was detected to compare the total number of retrogradely labeled cells to the labeled cells co-localized with 5-HT as identified using immunohistochemistry.

Injection Sites.

Initial injections targeting Piriform Cortex (PC) according to coordinates derived from *The Mouse Brain Atlas*[56], missed the target. Therefore, recalibration of the injection sites was necessary using different antero-posterior levels until optimal coordinates were confirmed. Brain slices were imaged for retrobead injections for Piriform Cortex (PC) at optimal coordinates (from Bregma, 2.5mm lateral; from midline, 2.2mm anterior; from dura 3.4 mm deep**) (Figure 10a)** and for Olfactory Bulb (OB) at optimal coordinates (from Bregma, 0.75mm lateral; from midline, 4mm anterior; from dura, 0.75mm deep) **(Figure 11a)** as was confirmed in *The Mouse Brain in Stereotaxic Coordinates*[56]. Coronal sections were obtained and imaged according to protocols laid out in the Methods section. Both PC and OB injections were effective and sufficient.

Retrograde Tracing.

Retrobead retrograde transport was effective for the PC injection as there was transport from the injection site to the mitral cell layer of the Olfactory Bulb **(Figure 10b)**, which is the major source of afferent input to the PC[24]. There was also effective retrograde transport from the PC to the DRN, specifically the medial portion of the DRN **(Figure 10c)**. Immunohistochemical staining for TPH (a serotonin specific marker) was effective and merged images show co-localization of retrogradely labeled cells with TPH positive cells.

It was previously shown that the PC receives both 5-HT and non-5-HT projections from the DRN, which is confirmed by the present results as ~80% of PC projecting DRN neurons were TPH positive **(**n=1 mouse**; Figure 12)**. These results also confirm previous retrograde

tracing studies that used cholera-toxin B as a retrograde tracer showing direct 5-HT and non-5-HT projections from the DRN to the PC and report ~70% of projection neurons as being serotonergic[18].

Retrobead retrograde transport was also effective for the OB injection **(**n=1 mouse**; Figure 11b)**. Immunohistochemical staining for TPH was effective, and merged images show retrogradely labeled cells co-express TPH and beads. While the proportion of 5-HT projection neurons was similar to that found in PC at ~85%, the total number of neurons was considerably smaller. One possible explanation for the low expression of labeled cells in the OB could be that the injection did not target the glomurular layer **(Figure 11a)**, which was shown to receive dense 5-HT input[46] such that a more precise injection could give more accurate and denser labeling of DRN cells.

Spatial distribution.

Next, the spatial distribution of PC projecting DRN neurons was determined. To do so, the number of PC projecting DRN neurons was counted in consecutive coronal brain slices and the number of cells was plotted against the anatomical location, measured as distance from bregma **(**n=3 mice**, Figure 13a,b)**. Unfortunately, these brains had poor immunohistochemical signal and therefore only the total number of projection neurons is considered regardless of their neurochemical identity. However, given the large proportion of 5-HT projection neurons **(Figure 12c),** these results likely reflect the distribution of 5-HT neurons as well. These figures show a clear bias in the distribution of PC projecting DRN neurons with most of the neurons located in the posterior caudal portion of the DRN. Additionally, as can be seen in **Figure 13c,** most of the neurons are located medially.

In conclusion, these results confirm that there are projections to each region originating in the DRN, and a majority of these cells are TPH positive. They also suggest that PC receives a much larger serotonergic input. Further analysis of the spatial distribution of these neurons within the DRN reveals a highly non-uniform pattern. This meshes well with the idea that the DRN rather than being a spatially uniform nucleus broadcasting a unitary signal, is actually highly heterogeneous, containg various subpopulations of spatially distinct groups of neurons

each with distinct projection pattern. Finally, it is evident that retrobead transport is effective and the method is useful for anatomical tracing of the serotonergic system.

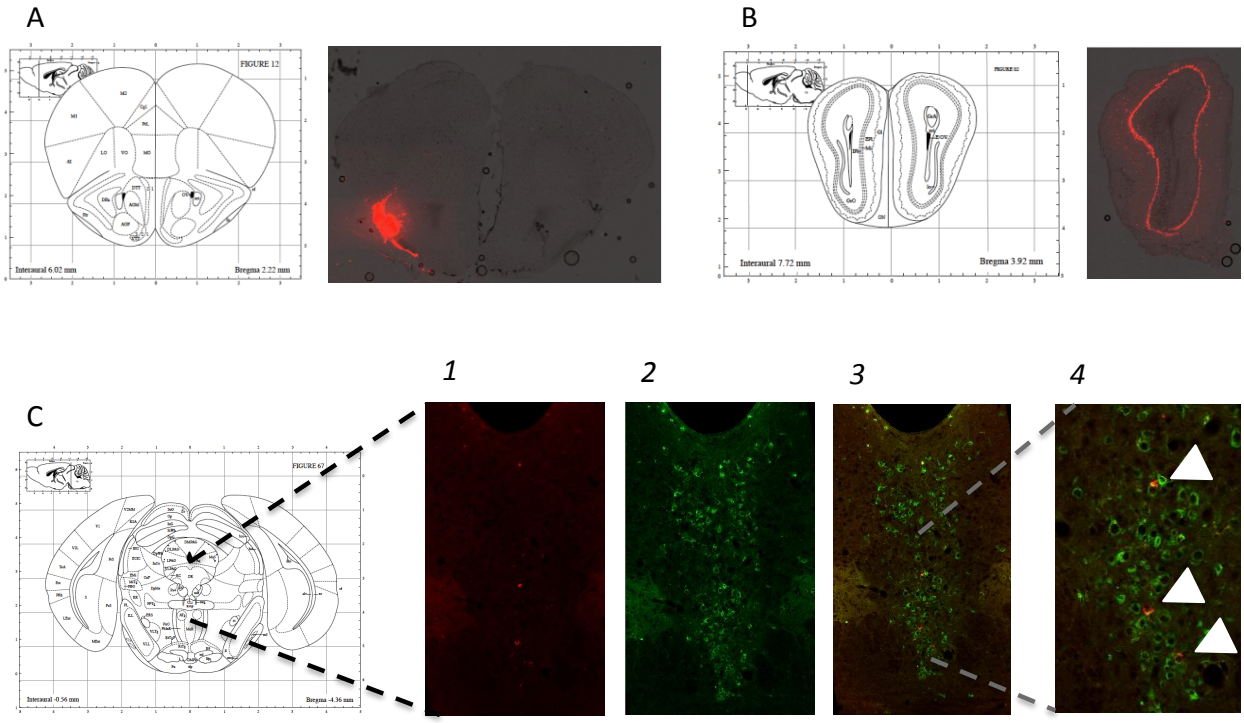


Figure 10. Retrogradely labeled cells from the PC injection site. A) (*left*) Atlas illustration depicting coordinates for Piriform Cortex injection site (from Bregma, 2.5mm lateral; from midline, 2.2mm anterior; from dura 3.4 mm deep); (*right*) Injection site of red retrobeads into PC. **B)** (*left*) Atlas illustration depicting coordinates for Olfactory Bulb; (*right*) mitral cell labeling in OB from retrobeads retrogradely transported from PC. **C)** (*left*) Atlas illustration with lines depicting DRN corresponding to images; (*1*) Red retrobeads showing DRN labeled cells that project to PC; (*2*) TPH staining for 5-HT cells in DRN; (*3*) Merged image depicting 5-HT expressing PC projecting cells; (*4*) Zoomed image of co-labled cells in DRN; white arrows show beads co-localized with 5-HT.

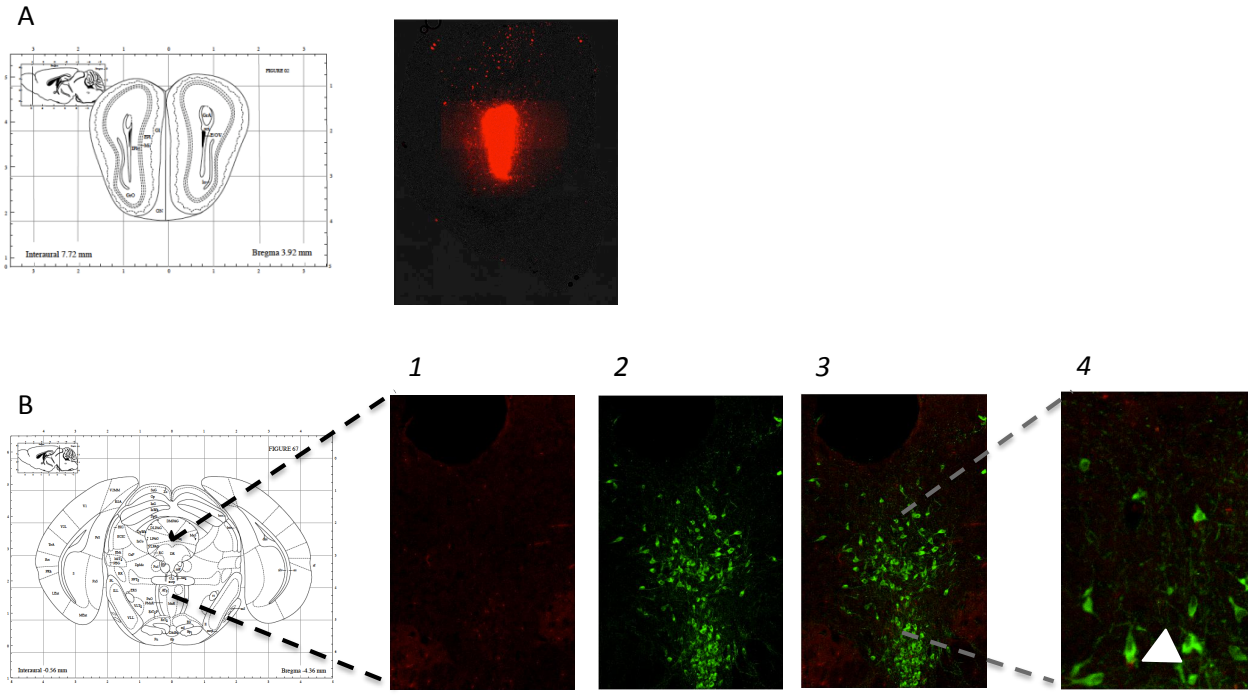


Figure 11. Retrogradely labeled cells from the OB injection site. **A)** (*left*) Atlas illustration depicting coordinates for Olfactory Bulb injection site (from Bregma, 0.75mm lateral; from midline, 4mm anterior; from dura, 0.75mm deep); (*right*) Injection site of red retrobeads into OB. **B)** (*left*) Atlas illustration with lines depicting DRN corresponding to images; (*1*) Red retrobeads showing DRN labeled cells that project to OB; (*2*) TPH staining for 5-HT specific cells in DRN; (*3*) Merged image depicting 5-HT OB projecting cells; (*4*) Zoomed image of co-localized cells in DRN; white arrows show beads co-localized with 5-HT.

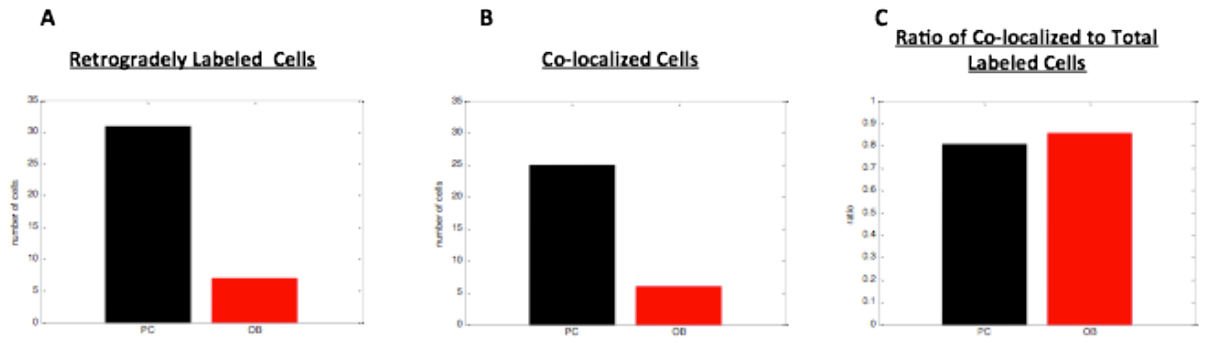


Figure 12. Cell count and co-localization of retrogradely labeled cells with 5-HT. A) Number of retrogradely labeled cells in DRN for Piriform Cortex (PC) and Olfactory Bulb (OB) from injections in two mice, one for PC and one for OB. **B)** Number of co-localized cells in DRN for each PC and OB. **C)** Ratio of total retrogradely labeled cells to co-localized cells in DRN from PC and OB.

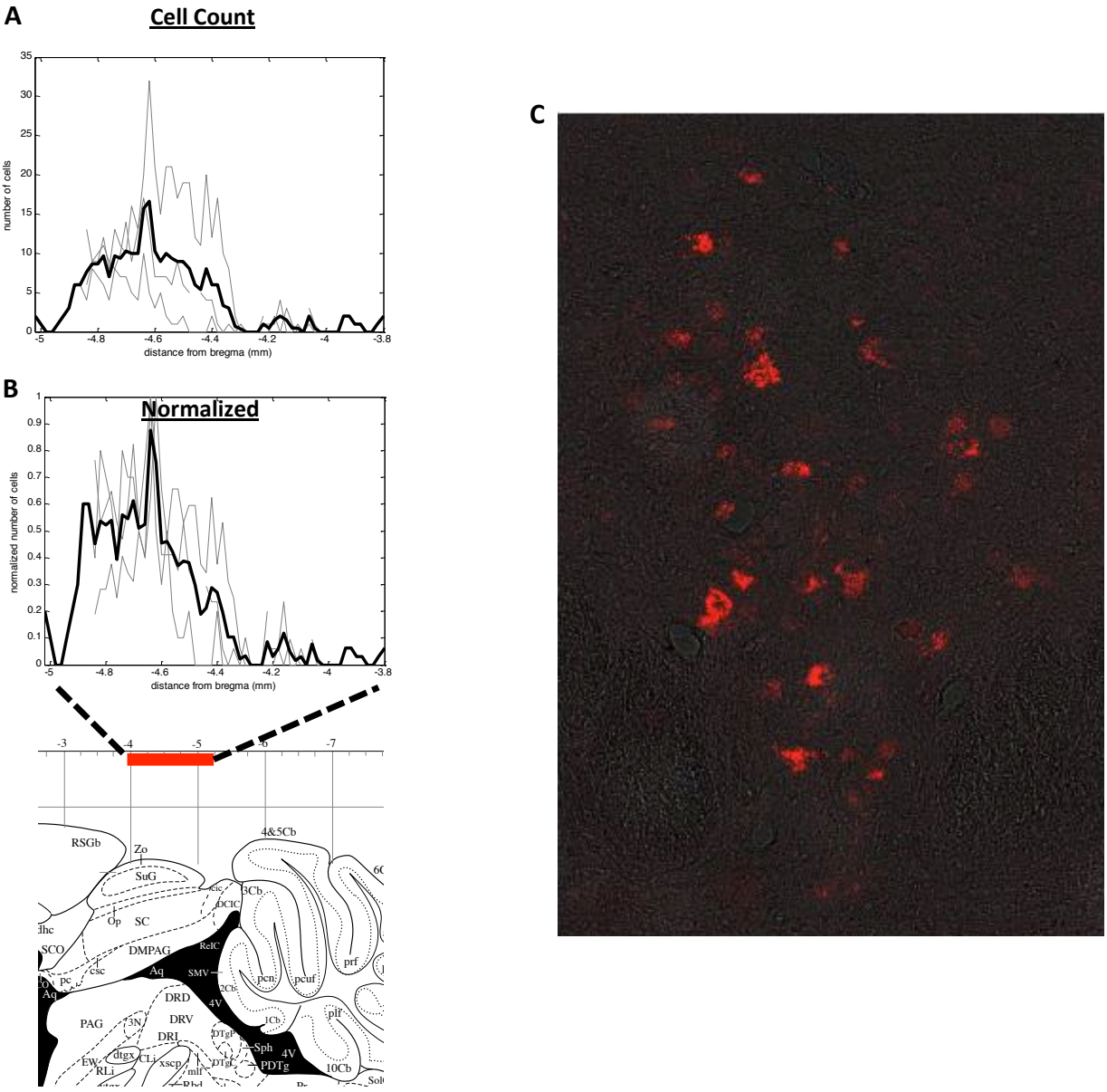


Figure 13. Spatial distribution of retrogradely labeled cells for PC injections. A) Average number of retrogradely labeled cells as a function of location, calculated as distance from Bregma (n=3 mice). **B)** Results in (A) were normalized by dividing the data from each mouse by its maximum. **C)** Zoomed image of retrogradely labeled cells in DRN from PC; image shows section from one mouse with highest count of labeled cells.

Viruses.

HiRet containing Pet1-Cre was injected into the PC of an ai32 transgenic mouse (Rosa26-LSL-ChR2H134R-EYFP) to assess virus viability and analyze transport to and localization in the Dorsal Raphe Nucleus. Importantly, since Cre is under the control of the Pet1 promoter, we expected expression of Channelrhodopsin-2 to be restricted to 5-HT neurons. CAV2 containing general promoter-Cre was injected into an ai32 transgenic mouse (Rosa26-LSL-ChR2H134R-EYFP) to assess virus viability and analyze transport to and localization in the Dorsal Raphe Nucleus. Using this construct with a non-specific promoter, we expected expression to be visible in the injection site, as well as any other regions which project to it. A total of 1µl was injected into the PC, and a three week waiting time was adhered to for transport and expression of the virus. Coronal sections were obtained and imaged according to protocols laid out in the Methods section.

Injections.

The injection sites were localized according to the coordinates optimized for the latex microsphere injections for Piriform Cortex (PC), and expression was seen locally in and near the PC, but no retrograde transport was visible. If the HiRet virus worked successfully, there should be no local expression since it contains a serotonin specific promoter (Pet1). Additionally, the expression pattern in the PC shows patchy patterns of eYFP expression not localized within cell bodies, which may indicate toxicity of virus and cell death in both cases. Prior waiting time of five weeks caused a similar effect to the cells in the injection site with the HiRet virus, therefore a three week waiting time was tested. The results obtained show either three weeks waiting time for expression may be too long for these viruses, or the amounts of viruses injected were too large. They also suggest that the Pet-1 promoter may not be specific enough and that leakage of expression may confound cell-type specificity with this construct. In conclusion, further experiments are needed in order to obtain efficient transport and expression of viruses from the PC to the DRN using these viruses or others mentioned in the Introduction section.

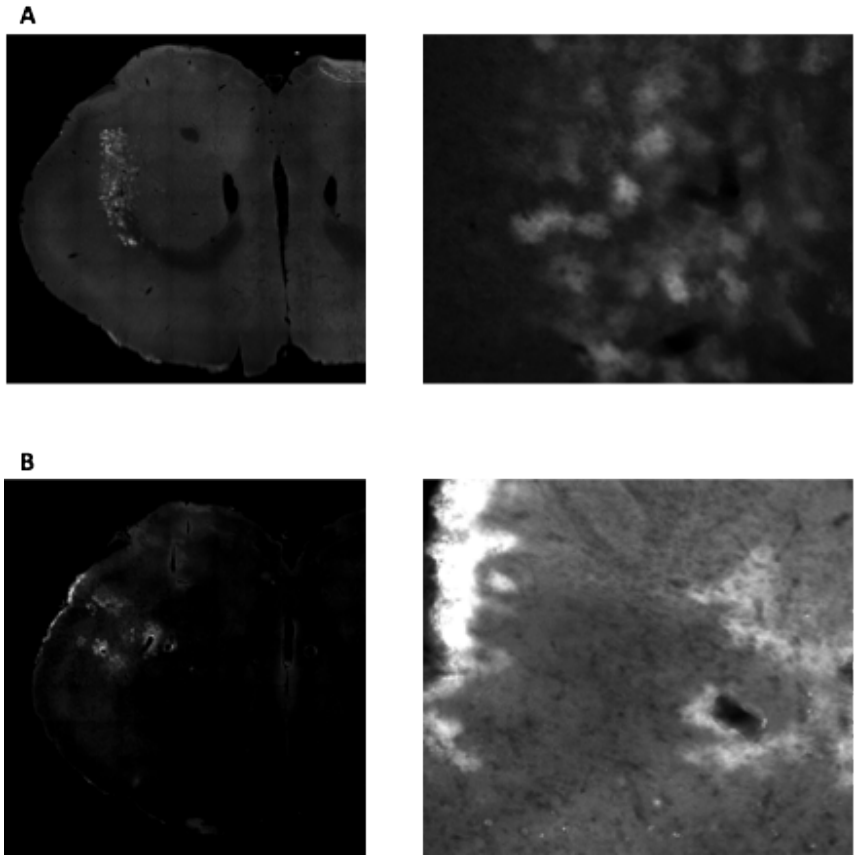
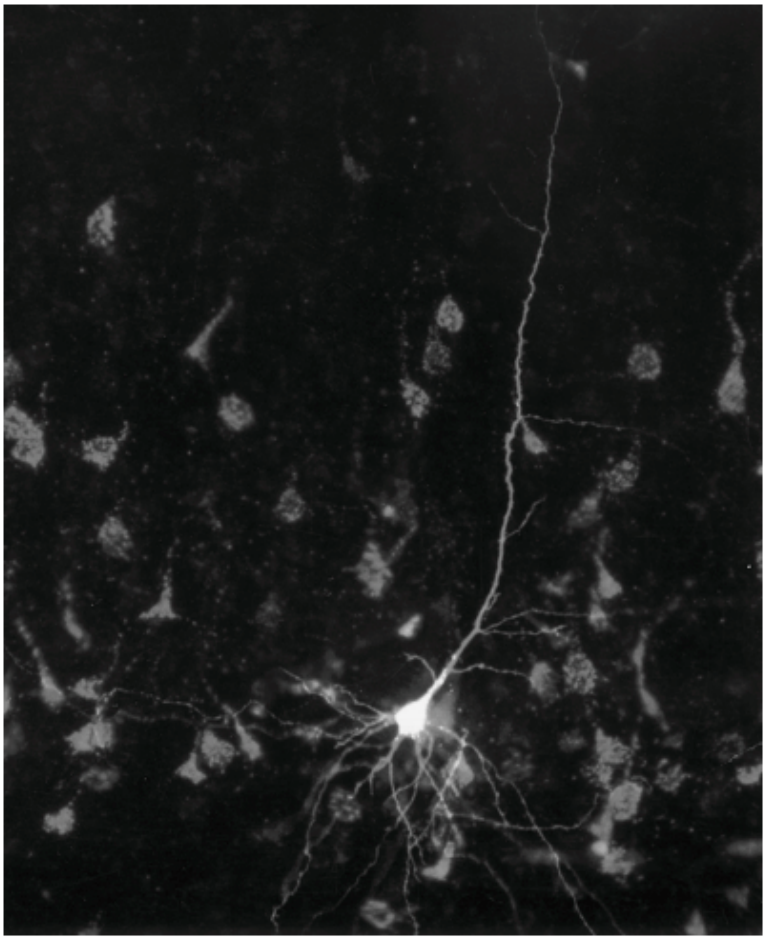


Figure 14. Virus Injection Sites. **A)** *(left)* Injection of HiRet virus into PC; *(right)* Zoomed image depicting infected cells in PC. **B)** *(left)* Injection of CAV2 virus into PC; *(right)* Zoomed image depicting injected cells in PC.

Chapter 4

Concluding Remarks



Conclusions.

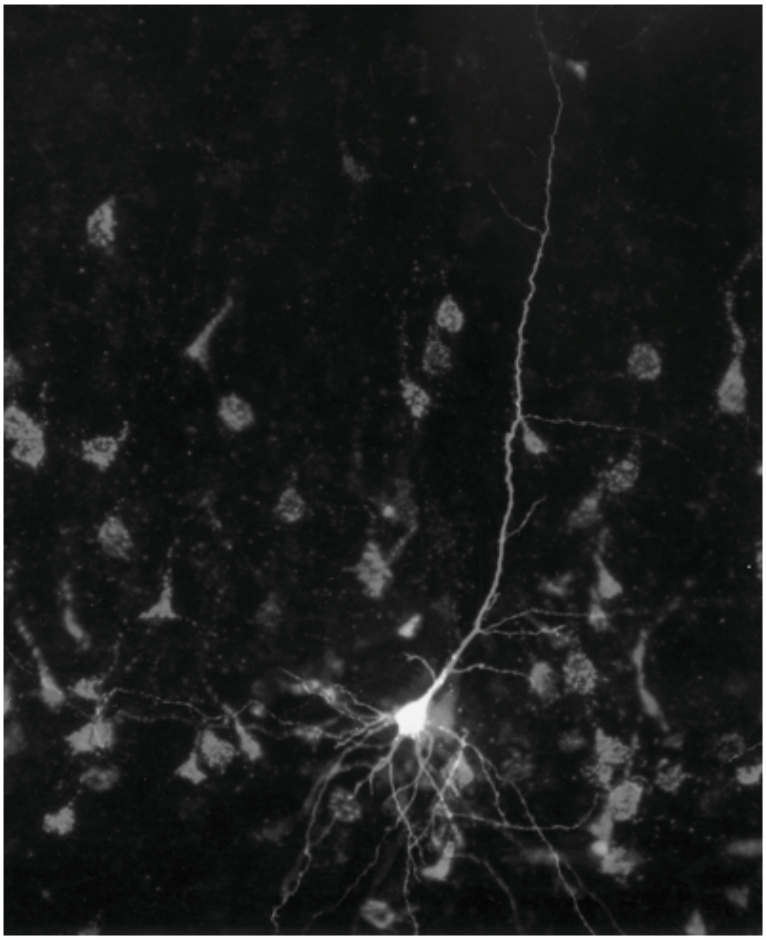
An obstacle to understanding the functional significance of 5-HT raphe neurons is the lack of knowledge of their precise postsynaptic targets[46], as well as the functional organization of the DRN itself. In this study, a number of tools were used to study structural and functional anatomy of the DRN and its projection to various parts of the central nervous system. We were able to optimize the experimental protocol for retrograde labeling using latex microspheres, specifically the precise location of the PC was determined and found to be different from the one found in the atlas. The results confirmed that there are projections from the DRN to both the PC and the OB, and a majority of these neurons are in fact 5-HT with PC projecting neurons being more numerous. These PC projecting cells were clustered in the posterior portion of the dorsal raphe region. More data needs to be collected and samples showing both accurate injection sites and optimal immunohistochemistry staining are necessary in order to confirm the exact proportion of 5-HT neurons projecting to the PC out of the entire population of PC projecting neurons. Additional experiments with co-injections of retrograde tracers to both OB and PC would test the possibility that some DRN neurons project to both regions simultaneously.

HiRet and CAV2 viruses were also tested, though it is still unclear as to the changes that need to be made in order for retrograde transport and clear expression to occur within the regions of interest. Optimization of the protocol is the first necessary step for using this technique in future retrograde and optogenetic studies.

Recent years have seen the emergence of novel tools aimed at studying behavior more specifically and with more precision (i.e., Optogentics), however this study demonstrated that in order to be successful, these tools need to be not only cell-type specific, but also projection-specific in terms of 5-HT organization within the DRN. Additionally, there needs to be a clearly defined layout of projections emanating from this region. This study could be developed further for future research, however with more time and precision, it could prove to be a successful experimental step that may inform many areas of serotonin research.

Chapter 5

References



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