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ANATOMICAL AND FUNCTIONAL CHARACTERIZATION OF SEROTONERGIC NEUROCIRCUITRY

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On the cover: Serotonergic circuit architecture represented using a voronoi pattern, made in architectural program Rhino 3D.
Credit: Dan Pollak Dorocic

Anatomical and functional characterization of serotonergic neurocircuitry

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The serotonin system arises from a small collection of brainstem structures called the raphe nuclei and innervates much of the central nervous system. A relatively small number of serotonin neurons modulate a wide variety of functions, from the basic such as homeostatic regulation of sleep and feeding to more complex roles in mood, motivation and emotion. Furthermore, serotonin signaling is implicated in the etiology and treatment of major depression, and is linked to numerous other mood and neuropsychiatric disorders. However, it has been difficult to assign a defined functional role to serotonin, possibly due to the heterogeneity of the neural population, including molecular, neurochemical, electrophysiological diversity and especially its broad connectivity.

The aims of this thesis are to examine the neuroanatomical circuits involving the major serotonergic nuclei, the possible heterogeneity of anatomical wiring including inputs and outputs of the serotonergic population, and connectivity with the basal ganglia structures of the brain.

In paper I, we characterize and quantify inputs to serotonergic neurons of the dorsal and median raphe nuclei on a whole-brain scale. We reconstruct whole-brain connectivity patterns by utilizing a genetic strategy for retrograde transsyaptic tracing of direct inputs to genetically-defined serotonergic neurons to uncover previously unidentified or disputed circuits, and provide functional confirmations of direct connections from forebrain regions to serotonergic cells. We characterize functional inputs from the prefrontal cortex, lateral habenula and basal ganglia. In paper II, we determine whether dorsal raphe serotonergic heterogeneity can be characterized by the input-output circuitry of the population. We describe the whole-brain presynaptic inputs to dorsal raphe serotonergic subpopulations that project to either the prefrontal cortex or striatum and contrast these findings with input-output circuitry of other cell types that interact with serotonergic neurons, including midbrain dopaminergic and dorsal raphe GABAegic populations. In paper III, we dissociate the functional connectivity between the different neuronal types of the striatum, a basal ganglia structure that receives serotonergic inputs and also projects to the dorsal raphe. We show that parvalbumin-expressing fast spiking interneurons in the striatum provide direct inhibition onto the projecting medium spiny neurons, which regulate the output to downstream basal ganglia structures, while avoiding other types of neighboring interneurons.

In summary, the work of this thesis provides a further step in untangling the heterogeneity of the serotonergic system, by targeting genetically-defined serotonergic neurons of the raphe nuclei and their afferent and efferent connectivity. Ultimately, knowledge of connectivity between genetically defined neuron types will be essential for modeling and understanding circuit function in health and disease.

LIST OF SCIENTIFIC PAPERS

I. **Pollak Dorocic I**, Fürth D, Xuan Y, Johansson Y, Pozzi L, Silberberg G, Carlén M, Meletis K.
A whole-brain atlas of inputs to serotonergic neurons of the dorsal and median raphe nuclei.
Neuron. 2014 Aug 6;83(3):663-78.

II. **Pollak Dorocic I**, Li ZE, Wahl F, Wang X, Xuan Y, Carlén M, Meletis K.
Input-output mapping of forebrain-projecting serotonergic and dopaminergic neurons.
Manuscript

III. Szydlowski SN*, **Pollak Dorocic I***, Planert H*, Carlén M, Meletis K, Silberberg G.
Target selectivity of feedforward inhibition by striatal fast-spiking interneurons.
Journal of Neuroscience. 2013 Jan 23 ;33(4):1678-83

Publications not included in the thesis:

Pozzi L, **Pollak Dorocic I**, Wang X, Carlén M, Meletis K.
Mice lacking NMDA receptors in parvalbumin neurons display normal depression-related behaviors and response to antidepressant action of NMDAR antagonists.
PLoS One. 2014 Jan 16;9(1):e83879.

CONTENTS

LIST OF ABBREVIATIONS

5HT	5-hydroxytryptamine, serotonin
AAV	Adeno-associated virus
ACA	Anterior cingulate cortex
ACB	Nucleus accumbens
ACh	Cholinergic
AP	Action potential
APH	Afterhyperpolarization
BLA	Basolateral amygdala
BST	Bed nucleus of the stria terminalis
CEA	Central amygdalar nucleus
ChR2	Channelrhodopsin
CLi	Caudal linear nucleus
CP	Caudate putamen, striatum
DA	Dopamine
dlSTR	Dorsolateral striatum
DR	Dorsal raphe nucleus
EGFP	Enhanced green fluorescent protein
FS	Fast-spiking
FST	Forced swim test
GABA	gamma-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GPe	Globus pallidus, external segment
GPi	Globus pallidus, internal segment
HRP	Horseradish peroxidase
IL	Infralimbic cortex
LH	Lateral habenula
LHA	Lateral hypothalamic area
LPGC	Lateral paragigantocellular nuclei
LTS	Low-threshold spiking

MR	Median raphe nucleus
MSN	Medium spiny neuron
SADΔG -EGFP(EnvA)	Rabies-EGFP
SERT	Serotonin transporter, Slc6a4 gene
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SSRI	Serotonin reuptake inhibitors
STN	Subthalamic nucleus
PAG	Periaqueductal gray
PFC	Prefrontal cortex
PHA-L	Phaseolus vulgaris-leucagglutinin
PV	Parvalbumin
PVN	Paraventricular nucleus
RG	Rabies glycoprotein
ROb	Raphe obscurus
RPa	Raphe pallidus
RMg	Raphe magnus
TH	Tyrosine hydroxylase
Tph	Tryptophan hydroxylase
TVA	Avian receptor complementary to EnvA envelope protein
Vgat	Vesicular GABA transporter
VGLUT3	Vesicular glutamate transporter 3
vmSTR	Ventromedial striatum
VTA	Ventral tegmental area
WGA	Wheat germ agglutinin

1 INTRODUCTION

The serotonin system has fascinated neuroscientists and inspired debates about its function in the brain for decades. There is a vast literature spanning anatomical, pharmacological and electrophysiological studies that have dissected the system at different functional levels. Although the serotonergic system is often described as unitary in the sense that the neurons comprising it all release the same neurotransmitter, it is becoming apparent that a large amount of heterogeneity exists and may account for the diversity of functions involving serotonin. This thesis will focus on the neuroanatomical circuits involving the major serotonergic nuclei, the possible heterogeneity of anatomical wiring including inputs and outputs of the serotonergic population, and links to the basal ganglia structures of the brain.

Serotonin is found in all the organs of the body, including the brain, skin, intestine, lung, kidney, liver, and more, and is present in nearly every living organism, including fungi, plants, and animals. It is intriguing that although the serotonin-releasing neurons in the mammalian brain are localized to a small, evolutionarily conserved structure of the hindbrain, and account for a relatively minute number of total neurons, they innervate and modulate virtually all parts of the brain. Therefore it is not surprising that the serotonergic system is involved in many basic functions including motor control, appetite and feeding, sexual behavior, mood and emotion, anxiety, reward and reinforcement, impulsivity, learning and memory, social behavior, and pain processing (Jacobs and Müller, 2010). Furthermore, the system is also implicated in a wide range of disorders, many encompassing the psychiatric realm, including depression, stress, drug addiction, obsessive compulsive disorders, schizophrenia, autism, attention-deficit hyperactivity disorder, panic and anxiety disorders, aggression, eating disorders and the underpinning of hallucinogenic drug action (Lucki, 1998).

Only recently has it become technically feasible to target and manipulate genetically-defined serotonergic neurons in the intact brain and during behavior. This advancement offers the possibility to define the heterogeneous serotonergic system into subtypes based on anatomy, molecular characteristics, and function, and ultimately determine the contribution of these subtypes to behavior.

1.1 SEROTONIN SYSTEM

The transmitter molecule was first discovered in 1937 as ‘enteramine’ in the neuroendocrine system by Erspamer and Viali, and later identified as serotonin (5-hydroxytryptamine, 5HT) (Erspamer and Asero, 1952). Soon thereafter, it was detected in the brain (Amin et al., 1954; Bogdanski et al., 1956) and hypothesized to play a role in behavior and mood (Woolley and Shaw, 1954). 5HT-containing neurons were visualized and localized to the brainstem, specifically to a midline cluster of cells defined as B1 to B9 (Dahlstrom and Fuxe, 1964). As a group, these 5HT-containing cell groups are referred to as the raphe nuclei and they innervate many diverse regions of the brain. The raphe nuclei are broadly divided into rostral

and caudal subdivisions, sending ascending projections and descending projections, respectively.

A large variation exists in 5HT neuron morphology, anatomical innervation patterns, neurochemical properties, electrophysiology, and gene expression. For instance, axons originating from the dorsal raphe nucleus contain fine beaded varicosities, while the median raphe nucleus projections contain large but sparse varicosities (Kosofsky and Molliver, 1987). In addition to being synaptically released, there is also evidence that 5HT can be diffusely released along the axon, constituting what has been termed volume transmission (Descarries and Mechawar, 2000; Fuxe et al., 2010). The diverse effects of serotonin are mediated by a variety of 5HT receptors, a group of mostly G protein-coupled receptors (and one ligand-gated ion channel), clustered into 7 classes and thus far 14 specific subtypes which can mediate both excitatory and inhibitory postsynaptic neurotransmission (Descarries et al., 2006).

Given the broad and diverse connectivity and physiology of 5HT neurons, it will be necessary to understand what 5HT does in the context of specific neural circuits. The following sections will outline specific aspects of 5HT circuit anatomy, interaction with other cell types, physiology as well as implications for behavior.

1.2 ANATOMY OF THE RAPHE NUCLEI

The rostral part of the raphe nuclei contains the dorsal raphe nucleus (DR) and median raphe nucleus (MR), which are the source of the forebrain-projecting 5HT neurons and the main focus of this thesis. Most of the anatomical and electrophysiological studies of the 5HT system have been performed in the rodent, in particular the rat, thus the focus of this section will be on the rodent 5HT system anatomy of the DR and MR.

The most rostral serotonergic nucleus is the Caudal linear nucleus (CLi), constituting the B8 group, which is additionally comprised of dopaminergic and substance P expressing neurons (Halliday et al., 1990b). Moving caudally, the MR appears, constituting B5 and B8 groups. It contains the second largest group of 5HT neurons, estimated at 25% of the total serotonergic population. The MR also contains peptidergic neurons including dynorphin, enkephalin and neurotensin (Björklund and Hökfelt, 1985).

The largest 5HT nucleus is the DR, a midline structure located in the ventral periaqueductal gray (PAG). It constitutes the B6 and B7 groups, and contains an estimated 50% of the brain's 5HT neurons. DR is situated ventral of the aqueduct and has been subdivided into the dorsomedial, ventromedial and lateral subdivisions (though more detailed subdivisions have also been proposed). Though the DR is the largest 5HT-containing nucleus, it is made up of a minority of 5HT neurons estimated at 30-50% (Descarries et al., 1982). Therefore, other cell types, including neurotransmitters and neuropeptides, contribute to the heterogeneity of the nucleus and will be discussed in the next section.

The caudal raphe group, which primarily sends descending projects, is comprised of three nuclei: the raphe magnus (RMg), raphe obscurus (Rob) and raphe pallidus (RPa) nuclei. The rostral and caudal groups are separated by a thin layer in the middle of the pons which lacks 5HT expression.

1.2.1 Efferent projections of DR and MR

Detailed anatomical studies of raphe projections have been completed, indicating the exhaustive innervation of the nuclei, however it is important to note that the majority of tracing studies were not restricted to 5HT neurons only, rather they labeled all cell types of the raphe and their efferents.

The DR 5HT system has extensive forebrain connections via two parallel pathways, the dorsal and ventral. Additionally, there is a less pronounced descending pathway projecting to the cerebellum, brainstem and spinal cord, however these will not be discussed in detail. The major targets of DR projections, moving posterior toward anterior, are the midbrain central gray, ventral tegmental area (VTA), substantia nigra-pars compacta (SNc), thalamic nuclei, amygdalar nuclei, lateral preoptic area, substantia innominate, ventral pallidum, striatum, bed nucleus of the stria terminalis (BST), and lateral septum. The cortex also receives dense 5HT innervation, including the piriform, insular, anterior cingulate, and infralimbic cortices (Vertes, 1991). Differences exist in projections from the rostral DR, which preferentially target the cortical regions, and the projections from the caudal DR, which preferentially innervate the hippocampus. Non-serotonergic neurons of the DR have also been shown to project to forebrain cortical and subcortical regions (Halberstadt and Balaban, 2007, 2008).

The MR 5HT system also projects to the forebrain, however it largely does not overlap with the DR projection targets. Instead, the MR neurons project along the midline, in particular to the medial mammillary body, supramammillary nucleus, hypothalamic nuclei, midline thalamic nuclei, zona incerta, lateral habenula, diagonal band nuclei, medial septum, and the hippocampal formation (Vertes et al., 1999). A seminal distinction between functional DR and MR projections was made in lesion experiments in rats, showing marked 5HT reduction in the hippocampus after MR lesion, while striatum and cortex transmission was not differentially affected, thus implicating MR as the major 5HT supplier to the hippocampus (Jacobs et al., 1974).

1.2.2 Topography of projections

There is strong evidence for a topographical organization of DR 5HT neurons based on projection target. Differential projections have been defined from the various raphe structures, including from the caudal raphe, and the MR and DR (Jacobs and Azmitia, 1992); from specific subregions of DR including the medial, ventral and lateral DR; as well as from rostro-caudal partitions of DR. Generally, rostral nuclei neurons tend to project to more rostral forebrain regions, compared to caudal DR neurons (Abrams et al., 2004). Within the DR, retrogradely labeled neurons that project to the prefrontal cortex (PFC) are localized to

the midline, while neurons projecting to nucleus accumbens (ACB) show a wider distribution also including the DR lateral wings (Van Bockstaele et al., 1993).

A more recent study utilizing genetic-targeting tools to specifically label 5HT neurons with Cre-dependent viral vectors revealed that different raphe nuclei have distinct terminal fields in their projection regions (Muzerelle et al., 2016). For instance, the ventral part of DR was shown to preferentially project to the orbital and agranular cortex, the central amygdala (CEA), and substantia nigra, the lateral part of DR projects to the lateral thalamic region, while the MR preferentially targets hippocampal regions **(Figure 1)**. Interestingly, these topographic rules seem to apply to both 5HT as well as non-5HT neurons, as the study using genetically restricted targeting found mostly similar patterns as older studies using indiscriminate labeling.

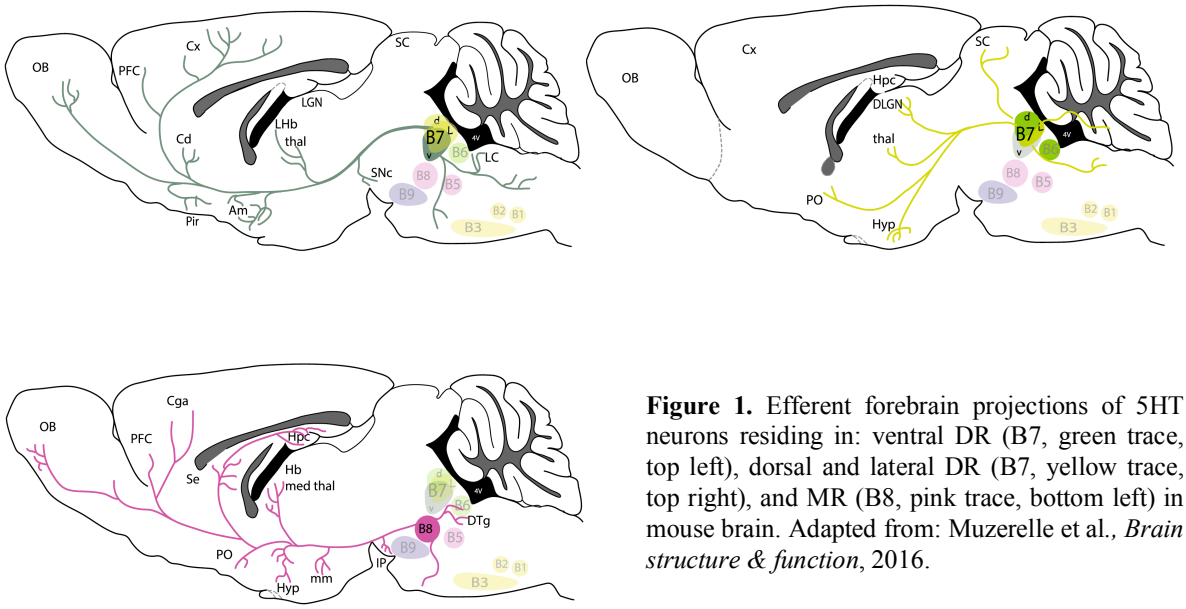

Figure 1. Efferent forebrain projections of 5HT neurons residing in: ventral DR (B7, green trace, top left), dorsal and lateral DR (B7, yellow trace, top right), and MR (B8, pink trace, bottom left) in mouse brain. Adapted from: Muzerelle et al., *Brain structure & function*, 2016.

1.2.3 Collateral projections

In addition to being topographically organized, there is evidence that individual DR 5HT neurons can have collateral, or branched, projections to anatomically separate regions (Abrams et al., 2004). Collateralization of DR projections to different target regions may provide an anatomic basis of how single 5HT neurons coordinate the modulation of specific but anatomically separate functional systems. The first branched DR projection observed collateralized to both substantia nigra and striatum (van der Kooy and Hattori, 1980), and since then several types of single DR neuron collaterals to various brain regions have been described. For example, single DR 5HT neurons can have projections to septum and entorhinal cortex, structures involved in hippocampal regulation (Kohler et al., 1982).

DR neurons have collaterals to the CEA and paraventricular nucleus (PVN), regions which are involved in anxiety and conditioned fear. In a retrograde tracing study, 7% of CEA and

PVN-projection neurons in DR colabeled with both tracers, and half of those were determined to be 5HT (Petrov et al., 1994). This collaterization may play a role in 5HT modulation of functionally related but anatomically separate regions.

A further example of functionally-relevant DR collaterals is to ACB and PFC. Collateral DR neurons were quantified to 15% of all ACB-projecting neurons and 24% of all PFC-projecting neurons, most of them expressing 5HT (Van Bockstaele et al., 1993). This is of functional interest, given that 5HT differentially modulates the PFC and ACB, and regulates dopamine release in both regions (Di Matteo et al., 2008).

A more recent study reconstructed single long-projecting vesicular glutamate transporter 3 (VGLUT3) expressing DR neurons, which mostly co-labeled with 5HT, and showed highly collateralized axon terminals in the forebrain (Gagnon and Parent, 2014). Single DR neurons were shown to jointly innervate the PFC and hippocampus, striatum and SN, and VTA and ACB, again demonstrating that the 5HT system can modulate proximal and distal targets via the same individual neuron. The number of axon varicosities in a particular terminal field is also relevant, as it is correlated with extracellular neurotransmitter concentrations and may indicate input strength to a particular region. The study above recorded a varied number of axon varicosities, ranging from tens to thousands, in the PFC and other brain regions.

1.2.4 Afferent projections to DR and MR

Given that DR and MR have largely distinct efferent projections to the forebrain, it is interesting to note that their afferents are largely overlapping. Both receive inputs from cortical regions classified as part of the limbic system; hypothalamic regions including the preoptic areas, lateral and dorsomedial nuclei of hypothalamus; the lateral habenula (LH), the midbrain and pontine central gray; and also caudal regions such as the laterodorsal tegmental nucleus, the caudal raphe nuclei, and locus coeruleus (Peyron et al., 1998; Vertes and Linley, 2008). Again, it is important to point out that these retrograde tracing experiments include inputs to both 5HT and non-5HT neurons of the raphe nuclei.

Anatomical and electrophysiological experiments have shown that PFC and LH inputs to DR act on 5HT neurons via local GABAergic interneurons. PFC stimulation results in fast activation of putative GABAergic DR neurons and slower inhibition of putative 5HT DR neurons, suggesting GABAergic local inhibition of 5HT neurons (Hajos et al., 1998; Varga et al., 2001). Electrical stimulation of the LH also activates putative GABAergic DR neurons and suppresses firing of 5HT, which can be blocked by GABA receptor antagonists (Ferraro et al., 1996). Finally, there is evidence that PFC and LH inputs converge on GABAergic DR neurons and thereby inhibit 5HT activity (Varga et al., 2003b).

In summary, the raphe nuclei are divided into subregions based on their rostro-caudal location, and the DR is additionally divided based on its medio-lateral and ventro-dorsal coordinate. A large body of literature has described the efferent and afferent projections, topography, and to some extent the collateralization of DR neurons. Importantly, most of the past studies were not able to target genetically-defined 5HT neurons directly, rather they

either described the heterogeneous DR population as a whole, or relied on post-hoc histochemical characterization of a subregion of the raphe nuclei.

1.3 DEVELOPMENTAL ORIGIN OF 5HT NEURONS

Although the details of 5HT development are beyond the scope of this thesis, it is interesting to consider the developmental lineage of 5HT neurons as it relates to their anatomical location in the adult brain, and as a way to subdivide the neuronal population in a more precise molecular manner. Briefly, the 5HT progenitor neurons reside in the embryonic hindbrain (rhombencephalon). During development, transverse constrictions subdivide the brain into neuronal segments which are called rhombomeres (Lumsden and Krumlauf, 1996). Serotonergic neurons are derived from rhombomeres 1, 2, 3, 5, and 6 (R1-3, 5-6), while R4 does not produce 5HT neurons (Cordes, 2005), creating the gap that separates the rostral and caudal raphe nuclei.

Based on intersectional genetic fate mapping, distribution of the five major serotonergic developmental sublineages was determined in adult anatomical locations: R1-derived 5HT neurons make up the DR 5HT population and partially contribute to the MR; R2- and R3-derived 5HT neurons also contribute to the MR; R5-derived 5HT neurons contribute to parts of the RMg, while R6-derived 5HT neurons are also located in RMg, and additionally contribute to the caudal raphe nuclei, including the ROb and RPa (Jensen et al., 2008) **(Figure 2)**. Population and single-cell RNA sequencing of the rhombomere-defined 5HT subpopulations show unbiased clustering into distinct 5HT neuron subtypes defined by a combination of developmental lineage and adult anatomy (Okaty et al., 2015).

5HT neuron subtypes defined by their lineage and anatomy can be functionally distinct. In particular, R2-derived 5HT neurons residing in MR have been shown to have distinct electrophysiological properties, *in vitro* responses to drug application, sensorimotor gating, and enriched levels of the gene *Met* which is related to autism. Knocking this gene out in 5HT neurons resulted in reduced social behavior (Okaty et al., 2015).

Therefore, defining 5HT neurons based on a combination of genetic profile, developmental lineage and anatomy may uncover further functional and behavioral phenotypes specific to a subgroup of 5HT neurons.

1.4 CELL TYPES OF THE RAPHE NUCLEI

Upon their discovery, the raphe nuclei were initially considered to be a purely serotonergic in terms of neurotransmitter type. However, over the years several neurotransmitters and well as a plethora of neuropeptides were shown to be present as well, and it is becoming clear that the neurons of the raphe can co-express several of these simultaneously, underlining the complexity of the neurochemical architecture of the structure.

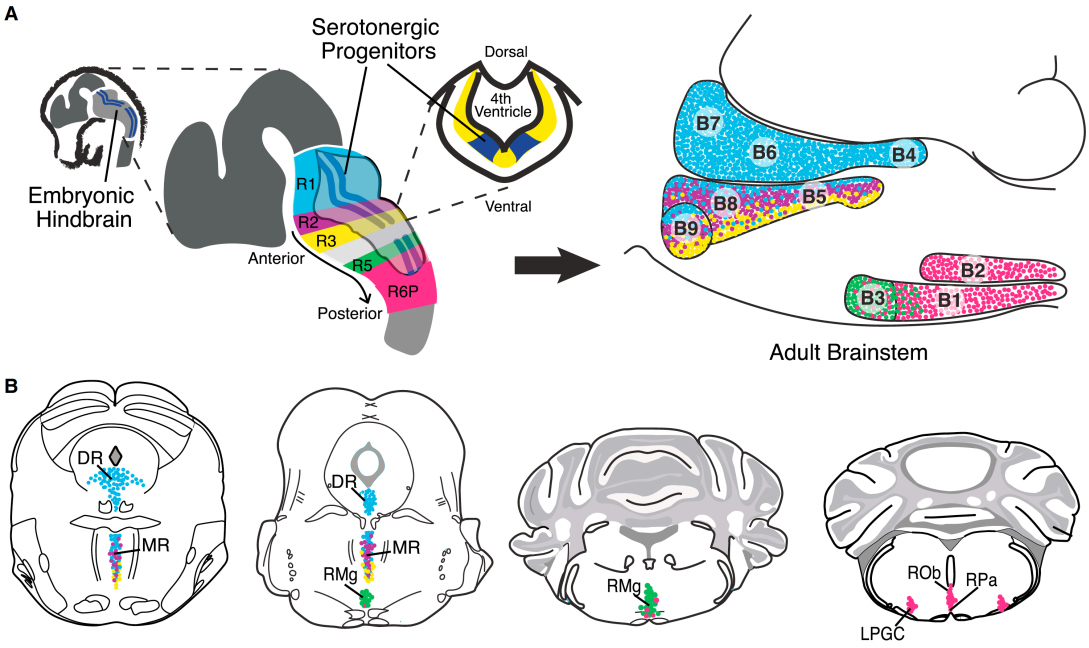

Figure 2. Anatomical distribution of 5HT precursor cells in the embryonic hindbrain rhombomeres (R1-3, R5-6) (A, left); and their fate-mapped distribution as mature 5HT neurons in the adult raphe (A right: sagittal plane, B: coronal plane). Adapted from: Okaty et al., *Neuron*, 2015.

One of the first additional neurotransmitters discovered in DR was dopamine (Lindvall and Bjorklund, 1974). Dopaminergic neurons are located in the ventromedial part of DR, and send projections to ACB, lateral septum, PFC and the striatum (Stratford and Wirtshafter, 1990). They also innervate structures of the extended amygdala, including CEA and BST, more so than other midbrain dopaminergic populations (Hasue and Shammah-Lagnado, 2002).

GABAergic neurons are partially intermingled with 5HT neurons in DR, but tend to be more localized in the lateral regions. They synapse onto 5HT neurons (Wang et al., 1992) thereby locally modulating 5HT neuron activity. GABA agonists applied locally inhibit 5HT neural firing and release (Gallager and Aghajanian, 1976; Tao and Auerbach, 2000). GABAergic dendrites and axon collaterals can project to other subregions of DR, or even contralaterally, indicating that individual GABA neurons can influence a potentially large numbers of dispersed 5HT neurons (Allers and Sharp, 2003). Limited amounts of co-expression of 5HT and GABAergic markers in single neurons have been reported (Belin et al., 1983; Fu et al., 2010), some of which send descending projections to the spinal cord (Hokfelt et al., 2000; Millhorn et al., 1987). A study estimated that 22% of lateral DR 5HT neurons co-express GABA, and suggested these neurons have separate neurochemical and electrophysiological properties from other 5HT DR neurons (Shikanai et al., 2012). DR GABA neurons have been chiefly considered to be local interneurons, however there is also evidence that they can send long-range projections to the forebrain (Bang and Commons, 2012). It is not known whether these long-range projection neurons release GABA in those forebrain structures and whether they co-express 5HT.

Glutamatergic neurons are also found in DR and there is strong evidence that they often co-express 5HT (Kaneko et al., 1990). A majority of DR and MR 5HT neurons also express VGLUT3 (Gras et al., 2002; Hioki et al., 2004). VGLUT3, together with the other isoforms VGLUT1 and 2, is a H^+-dependent carrier involved in fast excitatory glutamatergic transmission. VGLUT1 and 2 are restricted to known glutamatergic neurons widely expressed in cortical and subcortical regions, respectively, whereas VGLUT3 is expressed in neuronal populations not previously considered as primarily glutamatergic, such as 5HT and cholinergic neurons, making it the 'atypical' glutamate transporter (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002). Axons from DR and MR to the cortex co-release glutamate with 5HT (Hioki et al., 2004). Optogenetic stimulation of 5HT/VGLUT3 neurons in MR results in excitation of interneurons in the hippocampus (Varga et al., 2009). However, not all VGLUT3 neurons co-express 5HT, purely glutamatergic neurons projecting from MR to the hippocampus have been characterized as well (Domonkos et al., 2016; Jackson et al., 2009). Glutamate/5HT co-expressing neurons have been implicated behaviorally, especially in reward-related functions, and will be discussed in more detail upcoming sections.

Lastly, an extensive number of studies have determined the presence of neuropeptides expressed in the raphe nuclei. Co-localization of 5HT with neuropeptides varies between species as well as staining technique, nonetheless the following peptides are localized to the raphe and surrounding regions: enkephalin and dynorphin, substance P, cholecystokinin (CCK), neurotensin, vasoactive intestinal polypeptide (VIP), somatostatin, corticotropin-releasing factor (CRF), neuropeptide Y (NPY), galanin, and thyrotropin releasing hormone (TRH) (Fu et al., 2010).

1.5 ELECTROPHYSIOLOGICAL PROPERTIES OF 5HT NEURONS

1.5.1 *In vitro* electrophysiological characterization

Broadly, raphe neurons display slow, rhythmic activity (1-5Hz), broad action potentials (APs) as well as large afterhyperpolarization (APH) potentials. Recordings of putative 5HT neurons in slice offered an initial definition including: high-input resistance (150 – 400 MΩ), long action potentials (1.8 ms), large and slow AHP (10−20 mV, 200–800 ms), and 5HT1A receptor activation induced hyperpolarization (Aghajanian and Lakoski, 1984; Aghajanian and Vandermaelen, 1982), however these recordings were not confirmed histochemically. Later it was shown that many non-5HT neurons in DR display these same electrophysiological characteristics, including expression of 5HT1A receptor and inhibition by 5HT1A agonist (Beck et al., 2004; Kirby et al., 2003), thus highlighting that 5HT neurons cannot be recognized based on electrophysiology alone. DR and MR 5HT neurons differ in some aspects, namely a smaller time constant and larger AHP amplitude in MR 5HT neurons (Beck et al., 2004).

Further studies suggest that 5HT subpopulations residing in different subregions of the DR and MR differ in their electrophysiological profiles, including excitability, response to 5HT1A receptor activation, as well as morphological distribution of synapses and dendritic

fields (Calizo et al., 2011). Due to their intrinsic membrane properties 5HT neurons in the lateral wings of DR are more excitable than medioventral DR 5HT neurons, and display faster *in vitro* firing rates (Crawford et al., 2010), which may account for these neurons' sensitivity to stress. These findings show that 5HT neurons in different parts of DR have different cellular properties, such as ion channel distributions, which may also play a role in the heterogeneity of the population.

In addition, specific 5HT neurons display differential excitability and membrane properties based on their projection site, with basolateral amygdala (BLA)-projecting 5HT neurons having lower excitability and firing significantly less APs than PFC- and hippocampus-projecting 5HT neurons (Fernandez et al., 2016).

1.5.2 *In vivo* recordings of 5HT activity

Juxtacellular recordings of single neurons *in vivo* defined 5HT neurons as having broad APs (approximately 2 ms), slow firing rates (< 3 Hz) in a regular "clock-like" pattern, and inhibition to the 5-HT1A agonist (Allers and Sharp, 2003). The same study also described slow-firing non-5HT immunolabeled neurons that resembled 5HT electrophysiological characteristics, but had slower and less regular firing rates and narrower spike widths, though not sufficiently distinct for characterization purposes. Identified glutamic acid decarboxylase (GAD) immunoreactive neurons in DR were characterized as fast-firing (6 – 23 Hz), narrower spike width (1.1 ms), with a fluctuating firing frequency over time, morphologically smaller soma size and largely located in the ventral part of DR.

Although the activity of DR 5HT neurons is typically regular and "clock-like", ie. tonic firing, 5HT neurons can also display bursting activity (Hajos et al., 2007; Hajos et al., 1996). This bursting activity is characterized by spike trains consisting of single, slow and regular APs as well as spikes occurring in doubles or triples with a short inter-spike time interval (within 10-20 ms). Another study defined the stereotypically bursting 5HT neurons as having broad APs similar to clock-like neurons, but firing at slower rates and in a more irregular pattern (Schweimer and Ungless, 2010). The functional importance of this tonic and bursting activity has recently been explored in behavioral studies and will be discussed further below. Electrical stimulation of DR to induce burst firing enhances the release of 5HT in projection areas such as PFC, and increases neuronal activity of the regions, as compared to a single spike stimulation (Gartside et al., 2000; Puig et al., 2005).

A subset of 5HT neurons display a fast-firing pattern which is phase-locked to the hippocampal theta rhythm, the oscillation correlated to learning and memory formation (Kocsis et al., 2006). In anesthetized animals, electrical stimulation of MR desynchronizes spontaneous hippocampal theta rhythm, while lesion or pharmacological inhibition of 5HT neurons promotes continuous hippocampal theta (Vertes and Kocsis, 1997).

1.6 BEHAVIORAL CORRELATES OF 5HT NEURONAL ACTIVITY

In vivo electrophysiological recordings are crucial for identification of conditions responsible for 5HT neuronal activity and release. It is important to note that due to technological limitations, the majority of *in vivo* recording studies thus far have been performed either on putative 5HT neurons based on electrophysiological profile or with the low-yield juxtacellular labeling of recorded neurons followed by *post hoc* identification. Recently it has become possible to optically tag genetically defined 5HT neurons, or selectively transduce 5HT neurons with genetically encoded calcium indicators, thus allowing for large-scale *in vivo* recordings of unambiguous neural populations. These types of studies have begun shedding light on the activity and function of 5HT neurons specifically.

The first, and still the most robust, behavioral correlate found for DR neural activity is arousal state, with lowest activity during rapid eye movement (REM) sleep and highest during wakefulness (McGinty and Harper, 1976; Trulson and Jacobs, 1979), though not uniformly (Urbain et al., 2006). Behaviorally arousing sensory stimuli, including auditory or visual stimuli, activate DR neurons (Heym et al., 1982), as do locomotion and rhythmic movements (Fornal et al., 1996; Veasey et al., 1995).

In a study recording DR neurons during a behavioral task aimed at separating sensory, motor and reward variables with high temporal precision, neural responses were found to be transient and time-locked to specific behavioral events, however the firing patterns were variable and often responded to several types of events (Ranade and Mainen, 2009). Even when selecting for DR neurons with "classical" 5HT firing properties, no specific behavioral correlate was observed. These results point to the need for more selective probing of how specific variables relate to the anatomical and neurochemical diversity of the DR.

1.6.1 5HT involvement in reward and punishment

5HT neurons have been implicated in the processing of reward and punishment. An initial hypothesis that 5HT signaling is involved in negative affect or aversive information processing was proposed based on data from lesion, pharmacological and electrical stimulation studies. Aversive conditioning correlated with higher c-fos expression (Pezzone et al., 1993; Takase et al., 2004); putative 5HT neurons recorded in anesthetized rats can either be activated by noxious stimuli, in the case of tonically-firing subsets of neurons, or inhibited with short latency, in the case of phasically-firing neurons (Schweimer and Ungless, 2010); forced swimming alters 5HT levels in different projection areas (Kirby et al., 1995); depletion of 5HT enhances responding for reward (Fletcher et al., 1999); inhibition of MR 5HT neurons induces conditioning to obtain reward (Liu and Ikemoto, 2007); 5HT regulates behavioral inhibition (Soubrié, 1986; Wise et al., 1970); among others. In humans, systemic reduction in 5HT levels via acute tryptophan depletion (ATD) seems to lead to sensitivity to aversion and decreased behavioral inhibition (Cools et al., 2008; Faulkner and Deakin, 2014).

These results gave rise to the theory that 5HT has an opposing effect of the reinforcement regulated by dopamine (Boureau and Dayan, 2011; Cools et al., 2011; Daw et al., 2002;

Dayan and Huys, 2009). While dopamine is involved in behavioral activation and reinforcement to obtain rewards, 5HT could be involved in behavioral inhibition during expectation of punishment. This opponency theory predicts that unexpected punishment or omission of reward should activate 5HT neurons.

However, electrophysiological studies paint a more complicated picture, demonstrating that 5HT neurons can also be activated by reward. Self-stimulation experiments, which entail the animal performing operant responses for electrical stimulation, showed the first reinforcing effects of raphe stimulation (Miliaressis et al., 1975). Single-unit recordings of putative 5HT neurons in the primate DR showed tonic modulation by both the expected reward size, as well as delivery, with some neurons preferring either the large or small reward (Nakamura et al., 2008). This type of response differs from dopaminergic neurons which encode the reward prediction error, ie. the difference between expected and received reward (Schultz et al., 1997). Furthermore, the level of DR activity was shown to track progress during a task to obtain future rewards, with a subset of neurons showing sustained tonic excitation during entire trials leading to a reward, and a subset of neurons that showing tonic inhibition during trials leading to punishment (Bromberg-Martin et al., 2010). Thus, data suggests that putative 5HT neurons show tonic modulation for reward size, delivery as well as expectation for future reward. In a further primate study, rewarding and aversive outcomes were compared directly, by randomly alternating blocks of reward and punishment trials. About half of DR neurons discriminated positive and negative contexts by tonic modulation throughout the trail, additionally in the rewarding blocks the neurons fired phasically thereby coding reward values. It was concluded that single DR neurons encode both reward and punishment on short and long intervals, with tonic long-lasting coding of the reward and punishment blocks, and phasic short-lasting representation of positive trial value (Hayashi et al., 2015). Somewhat paradoxically, another study directly comparing the phasic firing of putative 5HT DR neurons in rat to reward or no-reward predicting cues showed that neurons displayed short-latency excitation preferentially to cues not associated with reward (Li et al., 2013).

The first study to unambiguously record genetically-defined 5HT neurons showed a heterogeneous neural response to reward and punishment on a short and long time scale (Cohen et al., 2015). Many of the recorded 5HT neurons displayed tonic firing modulation depending on the positive or negative state value on long timescales (across trail blocks) and 5HT neurons responded phasically to reward or punishments across short time scales (within the trail). The recorded neurons preferentially showed phasic excitatory activation to punishment trials.

A recent study causally linked 5HT neural activity to reward and reinforcement using optogenetic activation of ePet1-expressing neurons (Liu et al., 2014). Phasically activating 5HT neurons in DR caused several reinforcing behaviors including exploration of a stimulation-coupled spatial region, shift of sucrose preference to stimulation-paired water, enhanced operant self-stimulation, and the study also confirmed neural 5HT firing during reward-associated tasks. Interestingly, the study also investigated two separate components of

5HT neural signaling, namely the concurrent release of 5HT and glutamate. Both transmitters were shown to contribute to the reward signaling using knock-out and pharmacological assays, with a somewhat stronger effect on reinforcement behavior by glutamate. A separate study found inconsistent results for DR 5HT optogenetic stimulation. Activation of ePet-positive neurons in DR did not elicit rewarding or reinforcing behavior, as measured by self-stimulation and real-time place preference tests, nor did activation of dopaminergic or GABAergic DR neurons (McDevitt et al., 2014). However, specifically activating DR neurons lacking Tph2 (presumably glutamate-releasing) did elicit instrumental behavior, and these neurons were shown to strongly project to the VTA.

The DR to VTA projection has been further implicated in driving reinforcing behavior. VGLUT3-positive DR neurons projecting to VTA form excitatory synapses onto dopamine neurons projecting to ACB, and optogenetically activating this circuit induces ACB dopamine release and reinforces instrumental behavior (Qi et al., 2014). Therefore, the glutamatergic drive from DR 5HT-positive neurons seems to be strongly implicated in reward and reinforcement, however the sufficiency of 5HT is still not completely clear.

Recording neural activity of DR SERT-positive neurons in freely moving animals using photometry revealed that 5HT neurons can be activated by different types of rewards (ie. sucrose, food and social interaction) and are activated by unexpected reward but not punishment (Li et al., 2016; van der Worp et al., 2010). Opto-tagged 5HT neurons showed tonic followed by phasic firing properties to waiting and reward acquisition, respectively. DR GABAergic neurons were shown to be inhibited during reward seeking.

However, 5HT neurons were also shown to be activated by both positive and negative prediction errors, hence coding saliency or a surprise signal rather than valence, when recording the population activity of 5HT during reversal learning (Matias et al., 2016). In this study, 5HT neuronal activity was also slower to adapt to changes in reversal learning, compared to dopaminergic activity in the same task, suggesting a separate role of 5HT and dopamine neural firing in learning.

In summary, the role of 5HT signaling in reward and punishment is far from clear, though there is good evidence implicating 5HT activity in both positive and negative expectation and outcome, as well as state tracking over longer timescales. Methodological differences between studies should be taken into account. Older studies and primate recordings have been done on putative 5HT neurons based on their electrophysiological profile, a strategy that can result in false positives as well as exclude 5HT neurons with different firing properties (Allers and Sharp, 2003; Hajos et al., 2007; Schweimer et al., 2011). Optogenetic and photometry studies in transgenic animals either recruit or record activity of the whole genetically-defined targeted population, without taking into account nuances of individual cells (Li et al., 2016; Liu et al., 2014; Matias et al., 2016). And single-unit recordings of genetically-defined 5HT neurons offer the most detail and show the most heterogeneity, but are hard to extrapolate to the population due to small number of recorded neurons (Cohen et al., 2015). Nevertheless,

the methodologies offer valuable information for untangling the complexity of 5HT neural activity during behavior.

1.6.2 5HT involvement in waiting for reward

5HT is associated with behavioral inhibition and impulse control, with data showing that decreases in 5HT increase premature responding for reward and disinhibit behavior suppressed by punishment (Miyazaki et al., 2012; Soubrié, 1986). It follows that impulsive behavior can be a result of this disinhibition. 5HT depletion leads to preference of smaller, immediate rewards over larger, delayed rewards (Bizot et al., 1999; Mobini et al., 2000; Wogar et al., 1993). Both *in vivo* microdialysis and electrophysiological experiments in the rat showed DR 5HT levels and firing activity increase during the waiting time for a delayed reward, but do not change due to omitted reward (Miyazaki et al., 2011a; Miyazaki et al., 2011b). Causal evidence for 5HT involvement in patient behavior while waiting for reward was demonstrated by optogenetic activation of DR 5HT neurons. Both the frequency and the duration of patient trials was increased by activation of 5HT neurons (Fonseca et al., 2015; Miyazaki et al., 2014). However, the effect of 5HT stimulation in these experiments was not reinforcing, and therefore the enhancement in waiting is independent of a possible acute rewarding effect observed in the study mentioned above (Liu et al., 2014). Therefore, this data supports the theory that 5HT enhances long-term optimal behaviors and suppresses impulsive actions (Doya, 2002).

1.6.3 5HT involvement in mood and emotion

5HT is perhaps most famous for its link to mood disorders (Cools et al., 2008; Hensler, 2006), notably depression, as it is the main target of antidepressant actions of serotonin reuptake inhibitors (SSRIs) (Reid and Barbui, 2010). Several aspects of affect have been linked to 5HT and will be described below.

Stress is strongly linked to depression in humans (Hammen, 2005), and also has been associated with depression-related behaviors in animals (Katz et al., 1981; Strekalova et al., 2004). Uncontrollable stress activates DR 5HT neurons more than controllable stress, measured by cfos expression and microdialysis in both DR and specific projection regions including amygdala, PFC, and ACB (Amat et al., 1998; Bland et al., 2003a; Bland et al., 2003b; Grahn et al., 1999; Maswood et al., 1998). Stress sensitizes 5HT neurons and generates the behavioral changes characteristic of learned helplessness (Maier et al., 1995). The PFC – DR pathway was shown to play a role in evaluating whether the stress is controllable or uncontrollable (Amat et al., 2005). Optogenetic stimulation of excitatory PFC projections in DR results in increased behavioral effort in the forced swim test (FST) (Warden et al., 2012). These studies suggest that 5HT may be involved in inhibiting activity after uncontrollably stressful events.

5HT plays a role in aggression, a type of behavior related to impulsivity mentioned in the previous section. Increased levels of 5HT during development are associated with higher aggressive and impulsive behaviors (Cases et al., 1995; Ricci and Melloni, 2012), conversely

the opposite holds true in adults, with higher levels of 5HT decreasing these behaviors (Audero et al., 2013; Higley and Linnoila, 1997). Specifically, serotonin 1B receptor (5HT1B) is linked to both highly aggressive and impulsive traits (Rocha et al., 1998; Saudou et al., 1994). A recent study determined that 5HT can affect these behaviors through distinct circuits and during different time periods via 5HT1B action, and that rescue of 5HT1B expression in early post-natal development, but not in adulthood, lessens aggressive behavior (Nautiyal et al., 2015).

Understanding the direct role of 5HT neural activity in depression and anxiety-related behavior remains limited and conflicting. There is evidence that decreased 5HT contributes to depression-like behavior, including anhedonia, in animals (Bambico et al., 2009; Lira et al., 2003) and humans (Blier et al., 1990; Gos et al., 2008). Optogenetically and chemogenetically increasing activity of all 5HT neurons decreases depression-like behavior (ie. immobility in FST) but increases anxiety-like behavior (and also total locomotion) in mice (Teissier et al., 2015; Warden et al., 2012). This result corresponds to known effects of acute SSRI administration in humans and animals (Kurt et al., 2000; Sinclair et al., 2009). There is evidence that DR and MR activity plays a separate role in depression and anxiety (Lechin et al., 2006), with DR inhibition implicated in the depressive state and MR activity being more implicated in the anxiogenic state (Ohmura et al., 2014; Teissier et al., 2015). However, the DR 5HT – BST pathway is also implicated in driving the early anxiogenic effects of SSRI administration via corticotrophin-releasing factor (CRF) in BST (Marcinkiewcz et al., 2016). Overall, no cohesive set of data conclusively points to how 5HT contributes to affective disorders, emphasizing the need for more precise circuit deconstruction in terms of targeting specific 5HT subpopulations, possibly based on projection, molecular and genetic definitions.

1.7 INTERACTIONS WITH BASAL GANGLIA

The basal ganglia are an organized and interconnected network of subcortical nuclei, which are part of the cortico-basal ganglia-thalamic circuits and are involved in functions such as motor control but also more broadly in cognition and emotion. The basal ganglia contains several structures including the striatum (or caudate putamen, CP), internal and external globus pallidus (GPi and GPe), subthalamic nucleus (STN), and substantia nigra pars compacta (SNc) and pars reticulata (SNr). DR sends prominent 5HT projection to the basal ganglia, and modulates its activity by acting on various 5HT receptors.

The striatum is the primary input nucleus of the basal ganglia and is principally composed of inhibitory neurons, including the medium spiny projection neurons (MSNs) as its major population, as well as a diverse group of interneurons (Markram et al., 2004). Broadly, DRN stimulation and local administration of 5HT inhibits most neurons in the striatum (Davies and Tongroach, 1978; Olpe and Koella, 1977), however some excitatory responses in MSNs have also been reported (Park et al., 1982; Stefani et al., 1990; Vandermaelen et al., 1979). The response to 5HT is dependent on the expression of 5HT receptors in the release region. Presynaptic 5HT receptors, including 5HT1A and 5HT1B, are responsible for 5HT release in

the striatum and can also directly or indirectly influence the release of other neurotransmitters, such as glutamate from corticostriatal projections (Antonelli et al., 2005; Knobelman et al., 2000). Activation of the 5HT2 receptor family, particularly 5HT2C, directly inhibits striatal activity via MSN modulation (el Mansari and Blier, 1997; Rueter et al., 2000), as well as indirectly via activation of cholinergic interneurons, thus increasing the inhibitory tone of the striatum (Bonsi et al., 2007). Similarly, 5HT has been shown to activate fast-spiking interneurons, that are involved in feed-forward GABAergic inhibition of the striatal MSN projection neurons, also via 5HT2C receptor activation (Blomeley and Bracci, 2009).

Furthermore, 5HT exerts complex effects on the other basal ganglia structures, again dependent on the receptor involvement. Both activation and inhibition due to 5HT have been reported for STN neurons, while local application of 5HT or SSRIs results in excitation of GPe neurons via 5HT1B (Querejeta et al., 2005; Zhang et al., 2010b).

In the context of the basal ganglia, many studies have considered the involvement of 5HT in Parkinson's disease, a disorder that involves loss of dopamine neurons in SNc and results in motor control disturbances. Patients with the disease have lower levels of 5HT transmission due to the degeneration of DR in disease progression (Haapaniemi et al., 2001; Halliday et al., 1990a). Levels of 5HT and its metabolites are also decreased specifically in basal ganglia structures (Kerenyi et al., 2003; Scatton et al., 1983), while expression of some 5HT receptors is also affected (Ballanger et al., 2012; Fox and Brotchie, 2000). Interestingly, the decreased 5HT tone in Parkinson's disease patients has also be linked to increased prevalence of depression (Reijnders et al., 2008).

Lesions of dopaminergic neurons in parkinsonism animal models have resulted in conflicting results about the role 5HT in the disease. Studies have found increased, decreased and unchanged amounts of 5HT innervation and 5HT levels in the striatum after dopaminergic lesions, as well as either increased or decreased 5HT basal firing rates in DR (reviewed in (Miguelez et al., 2014)).

The standard treatment for Parkinson's disease is to transiently increase dopamine levels by administration of its precursor L-DOPA. However, long-term administration of the drug results in L-DOPA induced dyskinesias (ie. abnormal involuntary movements), a complication which has been linked to adaptive changes of the 5HT system. Reports have suggested altered levels of 5HT innervation to the striatum after L-DOPA treatment (Rylander et al., 2010). Moreover, L-DOPA can be converted into dopamine in 5HT neurons and fibers and then released in the striatum and other brain regions, a phenomenon not seen in normal non-L-DOPA treated animals (Arai et al., 1995; Hollister et al., 1979; Ng et al., 1970). Removal of 5HT afferents, or pharmacological inhibition of 5HT activity, results in elimination of the L-DOPA induced dyskinesias (Carta et al., 2007), further showing that the dopamine released by 5HT terminals is not therapeutic but rather a promoting factor for the induced dyskinesias. It is suggested that treatment with drugs that modulate 5HT

transmission, for example specific 5HT receptor agonists, may provide a therapeutic avenue for dyskinesias management (Munoz et al., 2008).

2 AIMS

The general aim of this thesis is to explore long-range and local circuits involving the 5HT system, the basal ganglia and their connectivity in a cell-type specific manner. The specific aims are:

1) To characterize brain-wide monosynaptic inputs to genetically-defined 5HT neurons of DR and MR, and to molecularly and electrophysiologically probe the identity of selected inputs including from the forebrain and basal ganglia (**paper I**).

2) To target DR 5HT neurons based on their projection region, either the striatum or PFC, and and map inputs to these differently-projecting 5HT subpopulations. To contrast the 5HT input-output connectivity patterns with connectivity of other cell types that interact with 5HT neurons (**paper II**).

3) To characterize the local microcircuitry in the striatum involving defined striatal cell types (**paper III**).

3 METHODS

Studying brain anatomy is a fundamental component of neuroscience research and has been a mainstay in the field since the days of Santiago Ramón y Cajal. The emerging field of neural connectomics is quickly advancing towards resolving complete neural circuit diagrams of model organisms. These wiring diagrams are vital in the quest to understand how neurons compute information, as they take into account the brain-wide inputs and outputs of defined neural populations. The advent of genetically-restricted transsynaptic tracing strategies allows for both dissection and functional manipulation of neural circuits in unprecedented detail. Furthermore, methods like optogenetics allow for causal investigations of circuit components in a cell-type specific manner. The research presented in this thesis utilized rabies-mediated transynaptic tracing and optogenetics for anatomical and functional dissection of the 5HT system.

3.1 TRANSGENIC ANIMALS AND VIRUSES

The ability to specifically target defined cell types is essential for the study of circuits. The main tools used in the research presented here are Cre recombinase driven transgenic mouse lines and Cre-dependent viruses carrying fluorescent markers or opsins.

Knock-in strategies can generate mice expressing Cre recombinase under the transcriptional control of, for example, the serotonin transporter (Slc6a4) promoter or tyrosine hydroxylase (TH) promoter, by placing the transgene downstream of the endogenous promoters (Lindeberg et al., 2004; Zhuang et al., 2005). Cre recombinase is an enzyme which executes site specific recombination between two DNA recognition sites (called Lox sites). In order to selectively target a cell type, it is important to choose a suitable transgenic mouse line and verify the specificity of Cre recombination. In adult mice SERT-Cre driven expression is restricted to 5HT neurons of the raphe, however TH-Cre expression may be unspecific in some midbrain regions, and therefore must be carefully validated *post hoc* using immunohistochemistry.

Viral vectors are used to introduce genes into the targeted Cre-expressing neurons via stereotaxic injections into the brain. In the experiments described here, adeno-associated viruses (AAVs) carrying fluorescent markers and/or opsins (light-sensitive proteins described further below) are used. The expression of these introduced genes is dependent on Cre recombinase in the targeted neurons due to the presence of a double-floxed inverted open-reading-frame (DIO) in the viral genetic code (Cardin et al., 2009; Zhang et al., 2010a). The gene to be expressed is situated between two sets of incompatible Cre recombinase recognition sequences and depends on Cre-Lox recombination. In the presence of Cre, the open-reading-fame is irreversibly inverted and is allowed to be expressed under the promoter present in the AAV (in our case the elongation factor 1-alpha (EF1α) promoter).

Thus, the combination of Cre recombinase transgenic mice specific for selected cell types (in the case of this thesis these are 5HT (SERT-Cre), dopamine (TH-Cre), GABA (Vgat-Cre) and

parvalbumin (PV-Cre)) and AAV viral vectors carrying fluorescent proteins and/or opsins under Cre-control, results in the ability to visualize and functionally probe selected cell types in the mouse brain.

3.2 RABIES-MEDIATED TRANSSYNAPTIC TRACING

We used a genetically modified rabies virus for anatomical labeling of presynaptic partners of a defined cell type. Rabies virus is a neurotropic virus which enters neurons via axon terminals, replicates in the infected cells, and subsequently passes to connected neurons though the synapse. Rabies virus has been shown to selectively travel in the retrograde direction, specifically infecting connected presynaptic neurons (Tang et al., 1999; Ugolini, 1995). The virus does not infect fibers of passage, nor does it lyse the infected cell to cause release and non-specific infection (Kelly and Strick, 2000). The wild-type virus is rendered replication-conditional by removal of the glycoprotein (RG), a protein necessary for transsynaptic spread, and replacement by green fluorescent protein (EGFP) (Etessami et al., 2000; Wickersham et al., 2007). The modified rabies virus is also engineered to target specific neuron types based on the avian receptor-ligand pair TVA-EnvA (SADΔG-EGFP(EnvA)) (Wickersham et al., 2007). By first introducing TVA receptor and RG via a Cre-conditional AAV virus (AAV-DIO-TVA-mCherry and AAV-DIO-RG) (Wall et al., 2010), only the selected Cre-expressing cell type in the targeted brain region is transduced by SADΔG-EGFP(EnvA) (from now referred to as Rabies-EGFP). Due to lack of RG in the presynaptic neurons, Rabies-EGFP is restricted to a single transsynaptic crossing, labeling the direct presynaptic population with EGFP **(Figure 3)**.

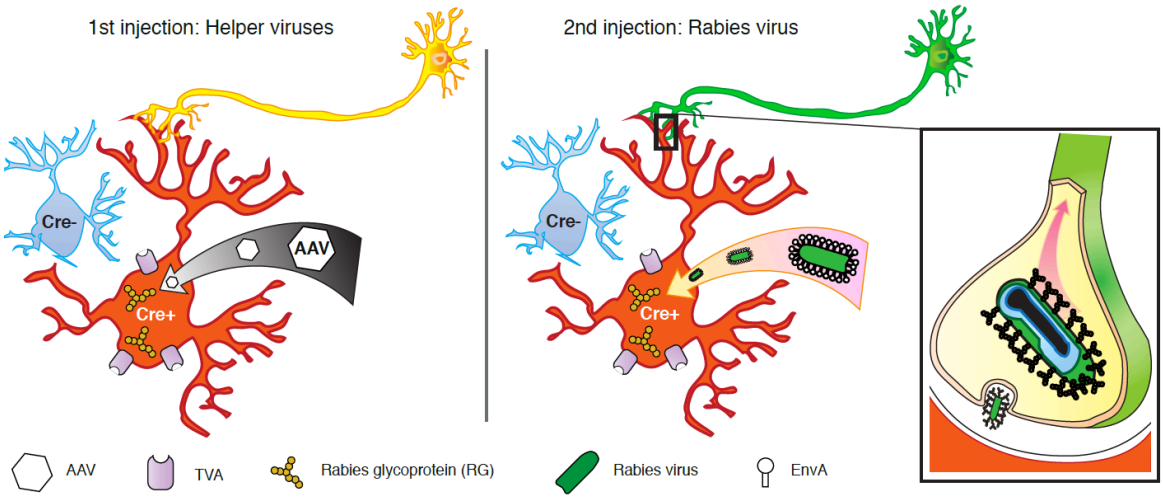

Figure 3. The first injection (AAV helper viruses) induces selective expression of TVA and RG in Cre-expressing neurons (Cre+). The second injection (EnvA-coated rabies virus) results in rabies uptake by TVA-expressing neurons and transsynaptic retrograde transport of RG-coated rabies virus into upstream input neurons. Adapted from Pollak Dorocic, et al., *Neuron*, 2014.

Mapping all inputs to a specific population is useful for delineating which cells give input to a whole population of interest **(Figure 4A)**, as in **paper I** where we mapped inputs to the whole 5HT population of DR and MR. It is also possible to pursue more detailed circuit tracing,

additionally taking into account the output of the starter population. The approach taken in **paper II** was to label forebrain-projecting 5HT and GABAergic neurons of DR and dopaminergic neurons of VTA and SNc with TVA and RG. Subsequently, the TVA-expressing axon terminals in the selected forebrain region were transduced with Rabies-EGFP which was transported back to the call body, and transsynaptically labeled the direct inputs of the projection-defined population **(Figure 4B)**. Thus, this approach allows for input-output mapping of a selected cell type localized in a specific brain area.

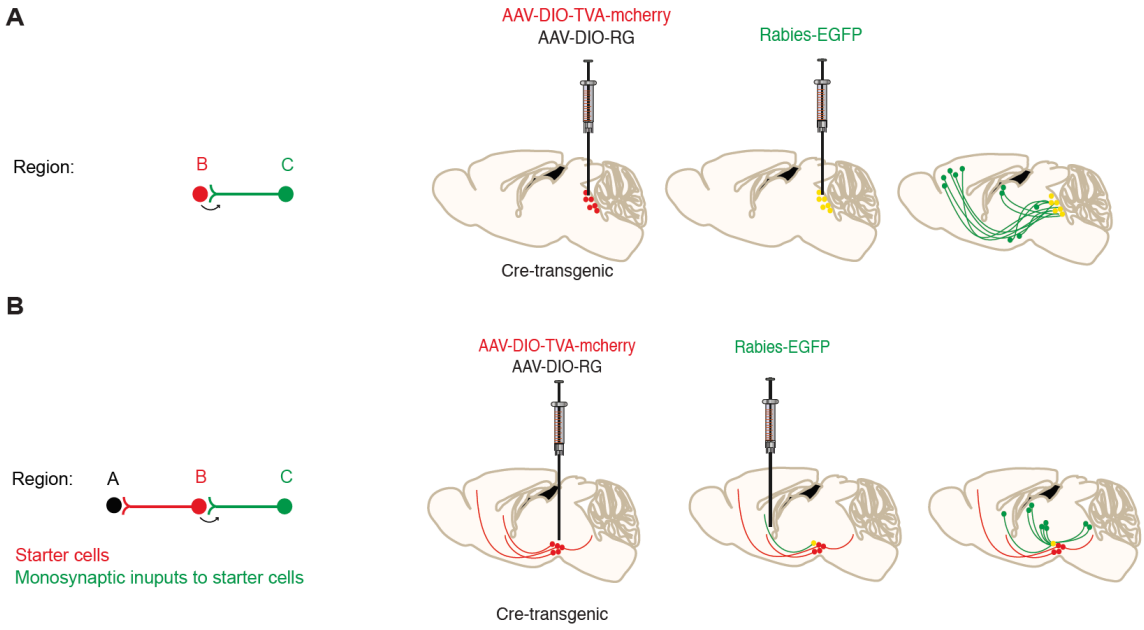

Figure 4. **A.** Tracing of monosynaptic inputs (from region C) to a genetically-defined starter population (in region B). **B.** Tracing of monosynaptic inputs (from region C) to a restricted genetically-defined starter population (in region B) defined based on their projection site (region A), ie. input-output mapping.

Pseudotyped, glycoprotein-deleted rabies virus has a number of advantages over conventional tracing strategies, chiefly being able to define the cell-type of the starting population targeted, rather than mapping connections between broad brain regions. Previously used non-viral tracers, such as horseradish peroxidase (HRP; (Kristensson and Olsson, 1971)), biocytin (King et al., 1989), *Phaseolus vulgaris*-leucagglutinin (PHA-L; (Gerfen and Sawchenko, 1984)), and others lack cell type specificity and may label axons of passage. Other tracers, such as wheat germ agglutinin (WGA) (Gonatas et al., 1979) or tetanus toxin C fragment (Schwab and Agid, 1979) can be expressed in a Cre-dependent manner, however they do not have a strong amplified signal making detection of weak connections difficult to quantify. They also cannot be limited to monosynaptic or polysynaptic spread. Conversely, Rabies-EGFP is strictly limited to the defined cell type in the targeted brain region and restricted to labeling only the direct presynaptic population. In addition, it has a high efficiency of labeling and can reveal sparse presynaptic neuron populations spanning large distances (ie. forebrain – hindbrain).

However, certain limitations exist with the use of rabies-mediated transsynaptic tracing. For example, it is unknown whether there are biases in regards to which synapses are crossed and if active versus inactive synapses or neurochemical identity influence rabies spread. Nor do we know the extent the labeling proportional to all presynaptic connections. Functional confirmation of active synaptic connections using electrophysiological recordings is necessary to corroborate anatomical findings. Additionally, control experiments are necessary to confirm that TVA does not infect non-Cre expressing neurons (by immunohistochemical identification of starter cells, and by injecting AAV-DIO-TVA and Rabies-EGFP into wild-type animals to confirm specific expression), and that Rabies-EGFP is truly lacking RG (by injecting Rabies-EGFP into Cre-animals expressing TVA only, while lacking RG, to confirm lack of transsynaptic spread).

In summary, rabies-mediated transsynaptic tracing allows for unambiguous labeling of genetically-defined starter neurons in the area of interest (identified by TVA-mCherry and Rabies-EGFP co-expression) and their brain-wide presynaptic partners (identified by Rabies-EGFP expression). In the paper I of this thesis, brain-wide mapping of input neurons to the 5HT population in DR and MR was performed. In paper II, brain-wide mapping of inputs to specific projection-defined 5HT and Vgat DR neurons and dopaminergic VTA and SNc neurons was performed.

3.3 OPTOGENETIC DISSECTION OF CIRCUITS

Optogenetics combines precise genetic targeting of a neural population with optical stimulation in a temporally precise manner (Boyden et al., 2005; Fenno et al., 2011). In combination with transgenic animals, genetically defined cell populations can be activated or silenced by using the light-activated microbial opsin Channelrhodopsin-2 (ChR2, activated by 470nm blue light) or Halorhodopsin (NpHR, activated by 580nm yellow light), respectively. New types of opsins are continually being developed for more precise neural manipulations (Berndt et al., 2016; Mattis et al., 2012). Optogenetics can be used for *in vitro* or *in vivo* modulation of neuronal activity on a millisecond timescale and over a wide range of frequencies.

In experiments of this thesis, we used ChR2-dependent optogenetic stimulation in brain slices in combination with electrophysiology. In **paper I**, to confirm that the monosynaptic connections to 5HT neurons revealed by Rabies-EGFP tracing make functional synapses, we introduced ChR2 to neurons in the selected forebrain brain regions (PFC and LH separately) under a general promoter (AAV-CAG-ChR2-GFP). 5HT neurons in DR of SERT-cre mice were visualized by expression of the fluorescent marker tdTomato. The forebrain-originating afferents were optogenetically stimulated while the response of tdTomato-expressing 5HT neurons was monitored via whole-cell recordings. Using this strategy, we were able to characterize whether the selected input onto 5HT neurons was monosynaptic, and whether it was excitatory or inhibitory by using pharmacological agents. In separate experiments, we targeted a genetically identified presynaptic population (D1-expressing neurons in striatum) by

injecting AAV-DIO-ChR2-mCherry into the ventral striatum of D1-Cre mice (Gong et al., 2007). We performed whole-cell recordings of putative 5HT neurons in DR during optogenetic stimulation of the labeled afferents **(Figure 5B)**. The neurons were categorized as 5HT by their electrophysiological profile, as well as *post hoc* colabeling for neurobiotin and the 5HT marker tryptophan hydroxylase (Tph). Light of 465 nm wavelength, 9 mW, and 200 ms pulses was applied to activate synaptic terminals connections (decreased to 5ms in case of strong synaptic responses). Thus, by optogenetic stimulation of defined afferent fibers, we were able to demonstrate functional excitatory or inhibitory monosynaptic connectivity of forebrain-localized neurons to DR 5HT neurons.

In **paper III**, a similar optogenetic approach was applied to dissect the microcircuitry of the striatum in slice recordings. We genetically targeted expression of ChR2 to PV neurons by injecting AAV-DIO-ChR2-mCherry into the striatum of transgenic PV-Cre mice (Hippenmeyer et al., 2005). Whole-cell recordings and optogenetic stimulation of ChR2-expressing PV interneurons resulted in activation of the targeted neurons, while the electrophysiological responses of the surrounding neurons were simultaneously recorded **(Figure 5A)**. Thus it was possible to characterize the response of different striatal neuron types, including MSNs, cholinergic interneurons as well as low-threshold spiking (LTS) interneurons, to PV activation.

In summary, we used optogenetic stimulation of both genetically-defined cell bodies and well as their afferent fibers in slice to characterize the postsynaptic effects of their activation.

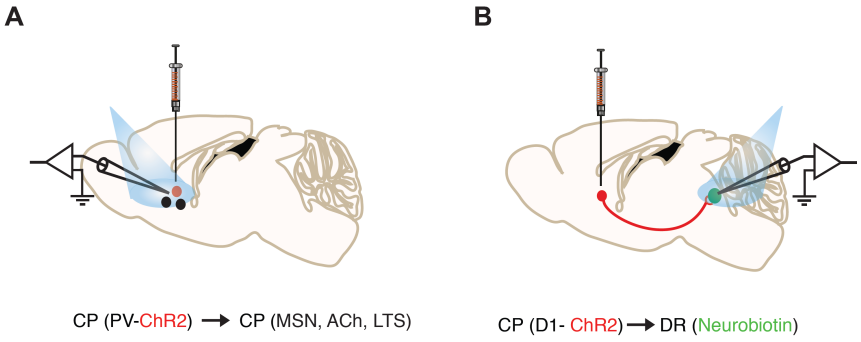

Figure 5. Optogenetic targeting of neurons in combination with slice electrophysiology. **A.** Activation of ChR2-expressing PV neurons in striatum, whole-cell recordings of neighboring cell type responses. **B.** Activation of D1 MSN ChR2-expressing terminals in DR, whole-cell recording of putative 5HT neurons, post hoc validation with antibody staining.

4 RESULTS & DISCUSSION

4.1 ATLAS OF INPUTS TO DR AND MR 5HT NEURONS

The main aim of **paper I** was to characterize and quantify whole-brain inputs to defined 5HT neurons of the two prominent raphe nuclei – the DR and MR. By utilizing a genetic approach to selectively target a modified rabies virus with transsynaptic retrograde tracing properties to 5HT neurons, we were able to perform comprehensive whole-brain quantification and localization of these direct inputs. Analysis of whole-brain connectivity uncovered previously unidentified circuits and provided several functional confirmations of direct connections from forebrain regions to 5HT cells of the DR.

4.1.1 Differences in inputs to DR and MR

In order to quantify and localize the EGFP-labeled input neurons, brain sections of 60 μm thickness including the forebrain, midbrain, and anterior portions of hindbrain were imaged and analyzed using a custom software which co-registers each section to the corresponding Allen Mouse Brain Reference Atlas coordinate (http://atlas.brain-map.org/). This allowed us to generate a comprehensive catalogue of the anatomical localization of each EGFP-labeled neuron to 553 out of 677 discrete regions defined by the Allen Reference Atlas. We found 80 distinct anatomical areas that contained an average of 100 EGFP-labeled neurons or more in the DR experimental group.

Our viral strategy revealed extensive brain-wide Rabies-EGFP labeling, and we quantified all labeled inputs to DR and MR. DR 5HT neurons received on average more inputs (59,710 ± 15,759, mean± s.d., 6 animals) than MR 5HT neurons (9,219 ± 2,481, mean± s.d., 4 animals). Given that DR contains more 5HT neurons than MR, we accounted for the different number of starter neurons and calculated that the ratio of the input population to the starting population was 72x for 5HT neurons in DR and 29x for 5HT neurons in MR.

Overall, comparing inputs to DR and MR 5HT neurons, a similar pattern emerged on a gross anatomical level. In general, 5HT neurons in MR received less inputs from PFC regions and displayed some preferential input distribution in anatomical subdivisions such as the hypothalamic medial zone (MEZ), superior colliculus motor-related (SCm), and zona incerta (ZI). The amygdala showed the largest difference in connectivity, with CEA preferentially innervating DR 5HT neurons, while having sparse connectivity with MR.

4.1.2 Whole-brain connectivity patterns

The anatomical areas containing most direct presynaptic inputs to the 5HT population were located in the mid- and hindbrain regions, specifically the periaqueductal gray (PAG), midbrain reticular nucleus (MRN), lateral hypothalamic area (LHA), pontine reticular nucleus (PRNr), motor related superior colliculus (SCm), substantia nigra pars reticulata (SNr), zona incerta (ZI), ventral tegmental area (VTA) and inferior colliculus external (ICe).

The hypothalamus provides among the highest numbers of input, in particular from LHA, tuberomammilary nucleus (TMN), and dorsomedial nucleus (DMH). It is known that the hypothalamic regions are involved in homeostatic regulation of basic physiological processes such as sleep, food intake, and body temperature, some possibly via the 5HT system (Celada et al., 2002). Subsets of EGFP-labeled neurons co-expressed several markers of hypothalamic cell types including: hypocretin (Hcrt) and melanin-concentrating hormone (MCH) in LHA, and vasopressin in paraventricular hypothalamic nucleus (PVH), while they did not co-express other hypothalamic markers such as TH and oxytocin.

Inputs from the amygdala were located mostly in the CEA, as previously reported (Peyron et al., 1998), and to a smaller extent in the basolateralamygdala (BLA) and medial amygdala nucleus (MEA). The bed nuclei of stria terminalis (BST), which is part of the extended amygdala circuit and is implicated in anxiety-related behaviors (Jennings et al., 2013; Kim et al., 2013) showed dense labeling of inputs.

4.1.3 Direct PFC and LH inputs to DR 5HT neurons

We found prominent input labeling in PFC, most of which was specific to layer 5 throughout the cortical regions. Particular anatomical regions with EGFP-labeled neurons included infralimbic (ILA), prelimbic (PL), anterior cingulate (ACA), orbital, and insular cortex **(Figure 6A)**. Previous studies suggested that the primary input from PFC is via local GABAergic neurons in DR, producing disynaptic inhibition of 5HT neurons (Hajos et al., 1998; Varga et al., 2001). We found that optogenetic stimulation of ACA axon terminals in DR resulted in monosynaptic, glutamate-dependent responses, confirming our tracing data and demonstrating the existence of a direct excitatory pathway from PFC that likely plays a role in regulating 5HT activity **(Figure 6B)**.

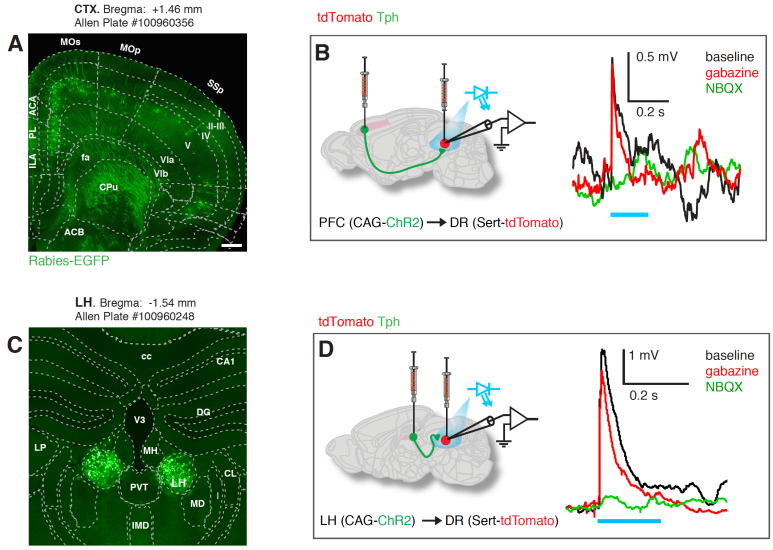

Figure 6. EGFP-labeled direct inputs from PFC (**A**) and LH (**C**) to DR 5HT neurons. Injection of CAG-ChR2 virus in PFC (**B**) and LH (**D**), whole-cell recoding of tdTomato-labeled 5HT cells in DR, traces show light evoked synaptic response. Adapted from: Pollak Dorocic, et al., *Neuron*, 2014

The LH is known to be a major provider of inputs to DR neurons (Peyron et al., 1998), but again it was suggested that this pathway primarily inhibits 5HT neurons via local interneurons in DR. Our experiments showed that LH provides dense direct inputs to 5HT neurons, and optogenetic activation of LH axon terminals in DR resulted in rapid depolarization of 5HT neurons that was glutamate-dependent **(Figure 6C-D)**. Thus, LH also provides direct excitatory input to 5HT neurons, revealing a parallel regulatory pathway in addition to the disynaptic inhibition of 5HT neurons. The functional role of this direct excitatory regulation of 5HT neurons by PFC and LH will be of interest to study in the future.

4.1.4 Direct basal ganglia inputs to DR 5HT neurons

A major discovery of the tracing experiments was the identification of direct inputs to DR 5HT neurons from the basal ganglia, which are so far not well described in literature. Numerous basal ganglia circuits contained EGFP-labeled neurons, including the striatum, globus pallidus and substantia nigra. A portion of the transsynaptically labeled neurons in the VTA and SNc co-expressed the dopaminergic marker tyrosine hydroxylase (TH), while the rest that did not likely correspond to the GABAergic population that is thought to project to the DR region (Kirouac et al., 2004). The EGFP-labeled input neurons in striatum (defined as caudoputamen (CP) and ACB) localized primarily to the ventral portion **(Figure 7A)**, including the ACB, and they displayed morphological characteristics typical of striatal projection neurons, the MSNs. The MSN identity was confirmed by marker FoxP1 staining, furthermore predominant lack of co-labeling with D2-marker preproenkephalin suggested most of these input neurons are D1-MSNs. Using optogenetic stimulation of ChR2-positive D1-MSN axon terminals showed synaptic responses in 5HT neurons which were blocked by a GABA-A receptor antagonist **(Figure 7B)**. This evidence points to a previously unknown striatal projection that monosynaptically inhibits 5HT neurons in DR.

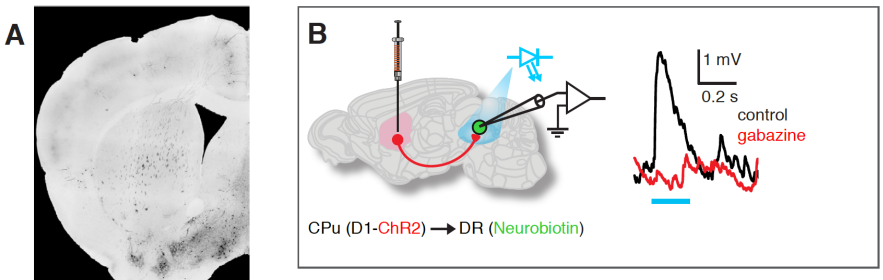

Figure 7. A. EGFP-labeled (black) direct inputs from ventral striatum to DR 5HT neurons. **B**. Injection of DIO-ChR2 virus in ventral striatum of D1-cre mice, trace shows light evoked synaptic response. Adapted from: Pollak Dorocic, et al., *Neuron*, 2014

In summary, paper I provides a comprehensive whole-brain classification of monosynaptic inputs that specifically target 5HT neurons in the DR and MR **(Figure 8A-B)**, with a special focus on elucidating chemical identities and/or electrophysiologically functional input population from the hypothalamus, PFC, LH, and ventral striatum. Ultimately, identification of

cell-type specific circuits, such as interaction of forebrain inputs to 5HT neurons, may lead to uncovering a role of different neuron types and circuits in generating behavior.

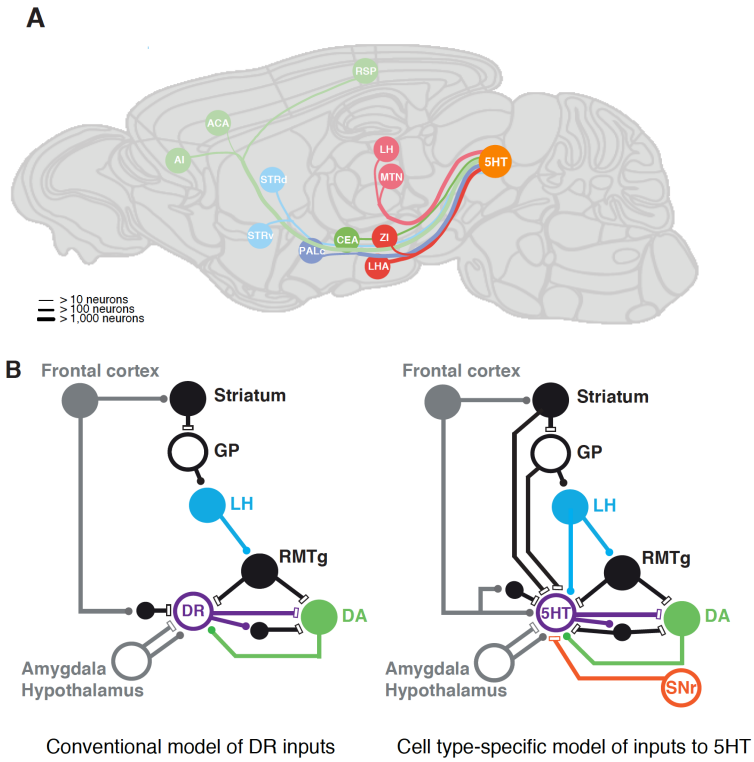

Figure 8. Summary of results from paper I. **A**. Localization and strength of inputs from selected regions to 5HT neurons. **B**. An updated circuitry model. Adapted from: Pollak Dorocic, et al., *Neuron*, 2014

4.2 INPUT-OUTPUT MAPPING OF FOREBRAIN-PROJECTING 5HT AND DOPAMINE NEURONS

The aim of **paper II** was to determine whether DR 5HT neural heterogeneity can be characterized by the input-output circuitry of the population. Distinct projection targets of 5HT neurons may define the functional heterogeneity of the population, while taking into account the presynaptic circuit organization of such projection-specific neuron populations could inform us about how 5HT neurons integrate incoming information and influence downstream targets.

Utilizing a modified protocol of rabies-mediated transsynaptic tracing, we describe the whole-brain presynaptic inputs to DR 5HT neurons that either project to PFC or striatum, and contrast these findings to input-output circuitry of striatum-projecting midbrain dopaminergic neurons, as well as striatum-projecting DR GABAergic neurons. We also identify a DR-projecting midbrain dopaminergic subpopulation that receives differential inputs compared to forebrain-projecting dopaminergic neurons.

4.2.1 Inputs to dopaminergic subpopulations of VTA and SNc

To validate our experimental approach, we first investigated whether the projection-specific rabies tracing strategy recapitulates the topographical organization of midbrain DA neurons with distinct ventromedial striatum (vmSTR) or dorsolateral striatum (dlSTR) projections

(Bjorklund and Dunnett, 2007; Lammel et al., 2008). We found that the majority of dopamine starter neurons in the vmSTR-projecting subpopulation were located in the VTA, while the dlSTR-projecting subpopulation of dopamine neurons was distributed more laterally in the SNc, as expected. We next went on to target DR-projecting dopaminergic neurons of the VTA.

We characterized the local topography of dopaminergic neurons in the midbrain based on their projection target and found DR-projecting dopaminergic neurons exhibited medial VTA localization. Additionally, the projection-defined dopaminergic subpopulations had a differential amount of local dopaminergic inter-connectivity, with the DA→DR subpopulation showing the highest percentage of local monosynaptic inputs that expressed the TH marker.

Next, we quantified extensive monosynaptic input onto vmSTR-, dlSTR- and DR-projecting dopamine neurons of the VTA/SNc. These subpopulations receive largely overlapping input, consistent with previous studies (Beier et al., 2015; Menegas et al., 2015). Our study is the first to characterize DR-projecting dopamine neurons of the VTA, and we showed that this subpopulation receives comparatively fewer inputs from forebrain regions, in particular from ACB and GPe **(Figure 9)**. However, DR-projecting dopaminergic neurons receive more input from LH compared to striatum-projecting neurons, and receive reciprocal connection from DR. It will be interesting to determine whether the DR input onto VTA dopaminergic neurons is 5HT-positive.

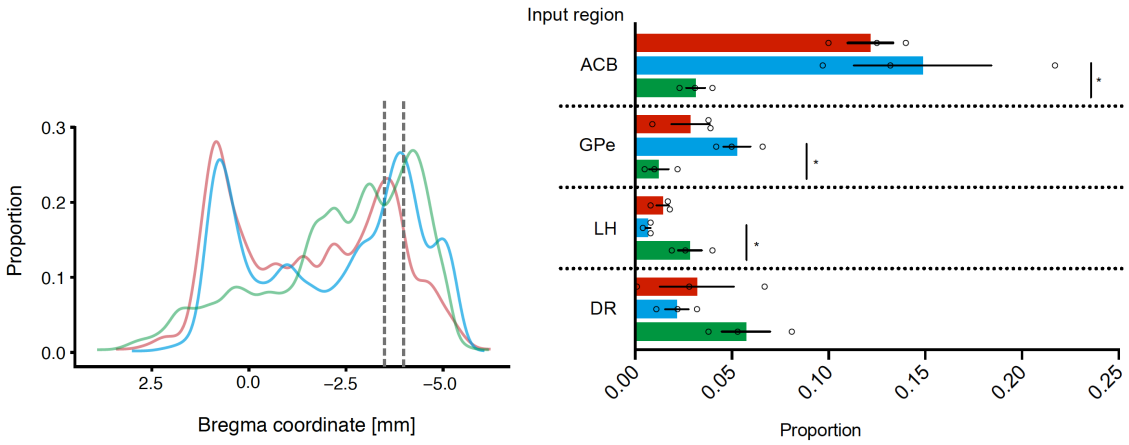

Figure 9. Left: Proportion of input neurons along the z-axis (bregma) for each tracing group. Bregma coordinate of VTA/SNc injection = -3.1 mm, VTA range depicted between gray dotted lines. Blue = inputs to dlSTR-projecting dopaminergic neurons, red = inputs to vmSTR-projecting dopaminergic neurons, green = inputs to DR-projecting dopaminergic neurons. **Right:** Comparison of inputs from nucleus accumbens (ACB), globus pallidus external segment (GPe), lateral habenula (LH) and DR to vmSTR- (red), dlSTR- (blue) and DR-projecting (green) dopamine subpopulations. Open circles denote each individual animal (n = 3 per group, mean ± SEM, $p<0.05$, one-way ANOVA for each input region, Tukey correction for multiple comparisons).

4.2.2 PFC- and striatum-projecting DR 5HT subpopulations

We applied the projection-specific viral tracing system to either PFC- or striatum-projecting 5HT neurons of the DR, in order to determine whether these groups of 5HT neurons receive

different sets of inputs, reflecting subpopulations of 5HT neurons that can be defined on their input-output structure.

Our tracing experiments show a similar topographical pattern as previously reported for ACB and PFC-projecting DR neurons (Van Bockstaele et al., 1993), with retrogradely-labeled striatum-projecting neurons being represented in higher numbers and having a wide expression pattern in the medial and lateral DR, whereas PFC-projecting neurons are more restricted to the midline. Local Tph-expressing EGFP-labeled input neurons were observed in both projection-defined DR subgroups, suggesting there is substantial 5HT interconnectivity within the DR.

Comparing the whole-brain quantification of monosynaptic inputs to PFC- or striatum-projecting 5HT populations showed that the two projection-defined groups receive largely overlapping input **(Figure 10A-C)**. The regions with the highest amount of input neurons are the periaqueductal gray (PAG), midbrain reticular nucleus (MRN) and pons (P), while forebrain regions proving input onto PFC- and striatum-projecting serotonergic neurons include striatum, ventral pallidum (VP), LH, LHA, medial and lateral preoptic areas (MPO, LPO), VTA, SNc, substantia nigra pars reticulata (SNr), and interpeduncular nucleus, among others.

Only few regions showed a difference in preferential input to PFC- and striatum-projecting 5HT neurons. The IPN was discovered to projects almost exclusively to the PFC-projecting serotonergic neurons, avoiding the striatum-projecting population. The LH also showed a trend for more numbers of inputs to PFC-projecting 5HT neurons **(Figure 10D)**.

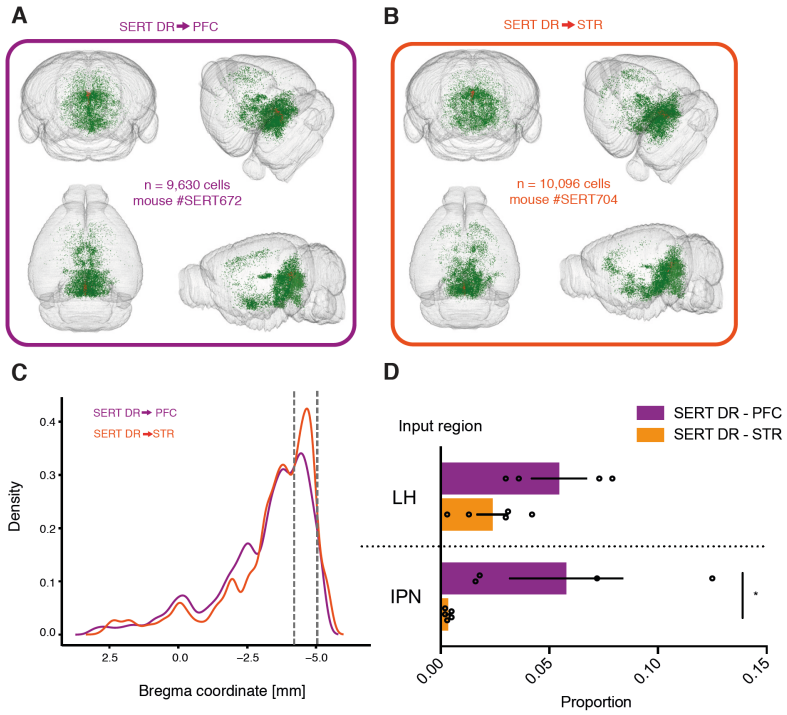

Figure 10. Whole-brain reconstruction of EGFP-labeled direct inputs to **A.** PFC-projecting and **B.** striatum-projecting DR 5HT neurons in one representative brain. **C.** Proportion of input neurons along bregma coordinate for the whole brain (dotted grey line represent dorsal raphe coordinates). **D.** Comparison of proportion of inputs from IPN and LH to PFC- (purple) and striatum- (orange) projecting 5HT subpopulations. Open circles denote each individual animal (n = 4 SERT - PFC group, n = 5 SERT - STR group, mean ± SEM, $p<0.05$, unpaired two-tail t-test).

4.2.3 Long-range projecting DR GABA subpopulation

In addition to the 5HT population, DR also contains substantial population of GABAergic neurons. We aimed to investigate whether DR GABAergic neurons have long-range projections similar to the 5HT neurons, and if so, to determine their input-output circuitry. The GABAergic neurons in DR were targeted using the projection-specific tracing strategy in Vgat-Cre mice and the vast majority of the starter neurons were determined to be GABAergic and not to co-express 5HT.

GABAergic neurons were found to have long-range projections to the striatum. Unlike 5HT neurons with striatum projections, which receive a broad set of inputs from a large number of cortical and subcortical areas brain regions, the striatum-projecting GABAergic neurons receive inputs from only restricted regions of the amygdala, namely the BST and CEA, as well as VP, LHA, and a limited number of mid- and hindbrain regions.

In summary, the experiments carried out in paper II show that 1) DR-projecting dopaminergic neurons of the VTA receive inputs preferentially from mid- and hindbrain regions but interestingly also from LH, compared to striatum-projecting dopaminergic neurons; 2) PFC and striatum-projecting 5HT DR neurons receive mostly overlapping input; and 3) DR GABAergic neurons have long-range projections to the striatum and receive input from a limited number of forebrain structures.

4.3 DISSECTING STRIATAL MICROCIRCUITRY

The basal ganglia are a group of subcortical structures that are interconnected and linked to functions including motor learning and action selection, as well as reinforcement learning and reward processing (Nelson and Kreitzer, 2014). A prominent basal ganglia structure is the striatum consisting mostly of projection neurons, the MSNs. As shown in paper I, D1-MSNs send direct inhibitory projections to 5HT neurons in DR. The functional significance of this projection is not known.

In **paper III**, we aimed to dissociate the functional connectivity of the various neuronal types of the striatum, including the small but diverse population of interneurons (Kawaguchi et al., 1995; Markram et al., 2004). The parvalbumin-expressing (PV) fast-spiking interneurons are known to form inhibitory synapses on both direct and indirect pathway MSNs (ie. D1- and D2-MSNs), thereby generating feedforward inhibition and potentially regulating the output to downstream basal ganglia structures.

Combining optogenetic activation of the genetically targeted PV cells in the striatum with whole-cell recordings, we showed strong GABAA-dependent synaptic inputs to MSNs. In addition, responses of other nearby interneurons, namely the cholinergic interneurons and low-threshold spiking (LTS) interneurons were recorded while PV-expressing interneurons were

optogenetically activated. Neighboring cholinergic interneurons did not show responses after optogenetically activating FS cells, while only a minority of LTS interneurons showed a weak response, suggesting neither population receives significant synaptic inputs from striatal PV neurons.

The results from this study showed that striatal PV interneurons form a feedforward inhibitory circuit that is target selective. PV interneurons inhibit MSN projection neurons, but do not functionally synapse onto cholinergic interneurons and only sparsely interact with LTS interneurons **(Figure 11)**. These results support the theory that MSN neurons are independently regulated by the different types of striatal interneurons. The local modulation by PV interneurons may have functional implications for downstream targets including the 5HT neurons of DR.

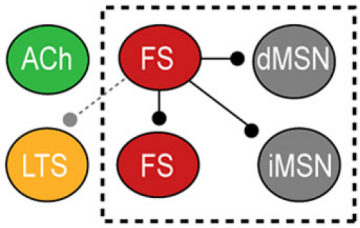

Figure 11. Schematic of striatal microcircuit connectivity. Ach = cholinergic interneurons, LTS = low threshold spiking interneurons, FS = fast-spiking parvalbumin interneurons, dMSN/iMSN = direct/indirect medium spiny neurons. Adapted from: Szydlowski et al., *Journal of Neuroscience*, 2013.

5 CONCLUSION & PERSPECTIVES

This thesis dissected the neurocircuitry of 5HT neurons arising from DR and MR. Tracing of inputs to genetically-defined 5HT neurons revealed extensive brain-wide presynaptic labeling. Our method allowed for the quantification of approximately 60,000 analyzed neurons per DR-targeted brain and localization to over 500 discrete anatomical regions, providing an unprecedented level of resolution.

In summary, we showed that inputs to DR and MR 5HT neurons are qualitatively similar, but quantitatively different, with considerably more inputs neurons targeting DR. Considerable numbers of presynaptic neurons were located in the hypothalamic areas, and in the case of DR-projecting inputs, showed a limited amount of overlap (10-20%) with well-known cell-type markers including hypocretin, melanin-concentrating hormone and vasopressin, likely accounting for regulatory input relevant for basic behaviors such as homeostatic regulation, sleep, food intake, and body temperature (Celada et al., 2002). Structures of the amygdala projected directly to 5HT neurons, specifically from CEA and BST, regions generally implicated in anxiety-related behaviors (Jennings et al., 2013; Tye et al., 2011).

We found new aspects of circuit organization involving PFC and LH projections to DR. Direct excitatory inputs from the PFC, a region generally implicated in top-down regulation of motivation, despair, and stress response (Warden et al., 2012; Amat et al., 2005) were discovered. We also found direct excitatory inputs from LH, a region linked to motivation and aversive processing. Both forebrain regions have previously been thought to interact with 5HT neurons via an intermediate inhibitory population made up of DR GABAergic neurons, thus in a disynaptic fashion (Varga et al., 2003a; Varga et al., 2001). Our data emphasizes the importance of parallel circuits (ie. mono- and disynaptic inputs from the same brain region) controlling 5HT neurons, which would be of interest to study further mechanistically and functionally.

We uncovered direct inputs from basal ganglia structures, including the GPe, SNc and SNr, VTA, and ventral striatum. The inputs from the VTA were shown to be both dopaminergic and non-dopaminergic. The VTA is implicated in reward processing and motivation, and projections from DR to VTA have been functionally linked to reinforcement. The functional relevance for the reciprocal VTA to DR connection has not yet been determined. The inputs from the striatum to 5HT neurons were defined as D1-expressing MSNs which exert long-range direct inhibitory influence on 5HT neurons. A limited amount of non-D1 marker labeled direct input was also detected.

We further tested the hypothesis that the 5HT population of DR can be subdivided based on projection target. Using a modified rabies virus tracing protocol, we were able to separately investigate the input targeting either PFC- or striatum-projecting 5HT neurons of the DR. We found mostly overlapping input regions and proportion of inputs to the two projection-defined

5HT populations. As an exception, the IPN was found to preferentially target PFC-projecting 5HT neurons while largely avoiding striatum-projecting 5HT neurons. The LH also showed a trend for preferential innervation of PFC-projecting 5HT neurons. Although the functional significance of such a shift in connectivity remains unknown, it is possible that it represents a circuit involved in motivation and decision-making, connecting subtypes of 5HT neurons with the PFC through IPN and LH regulation.

Although we found largely overlapping inputs to PFC- and striatum-projecting 5HT neurons of DR, we cannot rule out that a more anatomically distinct wiring of the 5HT population may exist. First, the PFC- and striatum-projecting 5HT neurons may be an overlapping population, since some of these neurons have been shown to have collaterals to both PFC and ventral striatum (Van Bockstaele et al., 1993). Targeting more anatomically distinct projection areas of DR, such as hypothalamus and amygdala, or LH and SN, may identify subpopulations of 5HT neurons that receive more distinct inputs. This would be of interest because these differently wired 5HT neurons may form functional subpopulations. Delineating the presynaptic circuit organization of these subpopulations could reveal how 5HT neurons integrate incoming information and influence downstream targets. Second, our tracing experiments showed variability in the proportion of inputs labeled. This may be due to differences in expression levels of TVA receptor in the 5HT axons in the projection region, and variability in the spread of the Rabies-EGFP after injection of virus. Utilizing different tracing approaches, such as the additional retrograde virus CAV2 to label the specific projection neurons (Schwarz et al., 2015) may prove to be more efficient at axonal expression, however this needs to be tested in the cell type and region of interest, as CAV2 may have differential tropism in terms of neural transduction.

Furthermore, we characterized a subpopulation of dopaminergic neurons of the VTA that project to DR. 5HT and dopamine are involved in functions related to processing of rewards and punishments, and the interaction of the two neuromodulators has been debated. Furthermore, both neurotransmitters are implicated in the etiology of mood disorders. An important step in defining the interaction of the dopamine and 5HT systems is the characterization of the anatomical interaction at the circuit level. Indeed, we show that the dopaminergic subpopulation in VTA projecting to DR has a distinct circuit architecture compared to forebrain-projecting dopaminergic neurons. DR-projecting dopamine neurons receive comparatively fewer overall inputs from forebrain regions, but more inputs specifically from the LH, in comparison to striatum-projecting dopaminergic neurons. The DR-projecting dopaminergic subpopulation also receives reciprocal connection from DR. It will be interesting to determine whether the reciprocal DR connection is 5HT-expressing and to determine its functional significance.

We show the existence of a GABAergic population in DR that sends long-range projections to striatum. There have been limited reports of long-range projecting GABAergic DR neurons, and not much is known about their circuit architecture or function. We found that these neurons

largely do not co-express 5HT, and they receive a limited amount of input from forebrain regions. GABAergic neurons in the DR have been primarily considered to act as local inhibitory interneurons that control 5HT activity through feed-forward inhibition (Varga et al., 2003a; Varga et al., 2001). GABAergic neurons in the DR have also been shown to be inhibited during reward seeking and activated by punishment (Li et al., 2016), and pharmacologically increasing their activity is associated with increased aggression (Takahashi et al., 2010). The functional significance of the striatum-projecting GABAergic neurons has not been determined, though the finding that these neurons receive considerable inputs from the extended amygdala, including CEA and BST, may suggest a role in emotional processing and anxiety-related behaviors.

Lastly, we dissected the microcircuitry involving the striatal fast-spiking interneurons. We show that genetically-defined PV neurons in the striatum provide direct inhibition onto MSNs, while neighboring ACh interneurons do not receive any synaptic PV inputs and LTS interneurons show only weak connectivity with striatal PV neurons. PV interneurons provide feedforward inhibition to both direct and indirect pathway MSNs (ie. both D1- and D2-MSNs) and are important in regulating the output to downstream basal ganglia nuclei (Planert et al., 2010). Interestingly, it is also known that DR sends 5HT projections to the striatum, and that 5HT activates striatal fast-spiking interneurons via 5HT2C receptor activation (Blomeley and Bracci, 2009). Therefore, it is possible that the PV interneurons are part of a feedback loop connecting DR 5HT neurons and the striatum. The study presented in paper III, in contrast to paper I and II, examines local connectivity in a cell-type specific fashion. Examining how long-range connectivity influences local neuronal interactions on a microcircuit level will be of importance to elucidate the influence of, for example, 5HT projections to forebrain regions and vice versa.

More broadly, it can be argued that the reason the 5HT system has been so elusive to study and to pin down functionally is because it has been considered and targeted as a unitary system. Due to technological limitations it has traditionally been difficult to target subsets of 5HT neurons based on, for example, their diverse connectivity or molecular properties. The main experimental avenue has been to combine electrical stimulation of the DR and pharmacological application of specific 5HT receptor agonists to the projection regions. Consequently, much is known about the contribution of 5HT receptor subtypes in 5HT signaling, but relatively little is known about distinct subpopulations of the 5HT neurons themselves, which display variability on several levels – gene expression, morphology, neurochemistry, connectivity and electrophysiology.

An emerging hypothesis is that the many functions modulated by the 5HT system are a result of specialized 5HT subpopulations driving distinct functions, or sets of functions. Thus, pharmacological treatment by drugs such as SSRIs or unspecific experimental targeting may in fact produce conflicting results due to their effects on a heterogeneous population. With the advent of methods allowing for targeting and manipulation of genetically-defined neural populations, it has become feasible to record, activate or inhibit these neurons during behavior.

Still, most studies thus far have indiscriminately manipulated or recorded the 5HT population as a whole.

The question then arises, which parameters should be taken into account to define 5HT neurons as functional subtypes? One recent approach has been to define 5HT molecularly, based on their genetic lineage, anatomical location and gene expression, into several molecularly distinct subtypes and link them to a particular set of cellular properties and functions (Jensen et al., 2008; Okaty et al., 2015). Furthermore, using a dual recombinase-based intersectional approach to selectively silence subsets of 5HT neurons based on co-expression of molecular markers has revealed functional 5HT subtypes that selectively modulate aggressive behavior (Niederkofler et al., 2016).

We propose to integrate another aspect in the cell type classification: whole-brain and local connectivity organization. We hypothesize that 5HT neural subtypes may be defined by the combination of their inputs, outputs and topographical localization within the raphe nuclei. The output organization of distinct projection targets of 5HT neurons can serve as a feature to define the functional heterogeneity of the population, while defining the presynaptic circuit organization of such projection-specific neuron populations could reveal how 5HT neurons integrate incoming information and influence downstream targets, and ultimately how this shapes behavior.

Utilizing genetic methods to select subpopulations of 5HT neurons, in combination with intersectional viral tools to target the efferents (with e.g. CAV or HSV retrograde viruses capable of transfecting projection-specific axon terminals) or afferents (with e.g. rabies-mediated transsynaptic tracing), will allow for selective targeting of 5HT neurons based on their connectivity, and study their function during behavior.

In addition, it will be crucial to generate molecular markers for more specific cell-type targeting of 5HT neural subtypes. For example, elucidating single-cell RNA profiles of 5HT neurons with distinct connectivity patterns may uncover gene expression differences and allow for generation of tools for targeting and functionally studying these populations.

Finally, a more specific molecular and anatomical definition of 5HT subtypes can serve as the foundation for identifying candidate pathways for pharmacological targeting. Current treatments for most psychiatric disorders, including mood disorders, have significant limitations in terms of efficacy and side effects, and progress in finding new therapies has been stagnant. Elucidating the neural circuit dysfunctions that contribute to the symptoms of major depression, as well as uncovering the contributions of specific neuronal cell types that participate in the regulation of mood and motivation may lead to new treatment strategies.

In this context, the work presented here takes the first step in defining brain-wide circuits targeting defined 5HT neurons, and uncovers an even higher degree of complexity than seen

before. The mapping experiments provide a blueprint of candidate circuits for further study in order to decipher the elusive 5HT system.

6 SUMMARY FOR NON-SCIENTISTS

The brain is a complex - and arguably least understood - organ in the body. The human brain is composed of over 80 billion neurons and trillions of connections that neurons use for communicating with each other. The overall 'chatter' of these billions of neurons results in the body being able to control basic functions necessary for survival, but also gives rise to our perceptions, thoughts and emotions. Most fundamentally, each neuron receives inputs from many other connected brain cells, and based on the strength and timing of these inputs, it computes a signal to pass on to the next connected neuron, forming the basis of neural communication. Neurons exist in a variety of types and communicate in several different ways. For example, excitatory and inhibitory neurons use fast transmission via tiny electrical currents, while so-called neuromodulatory neurons communicate on a slower time scale with the release of different types of chemicals. Making things a bit more complicated, the same neurons can operate using both the fast and sow mode of communication.

The research presented in this thesis focuses on one of the main neuromodulatory systems, namely the neurons that release the chemical serotonin. Neurons synthesizing serotonin are largely located in a network of small structures deep within the brain, called the raphe nuclei. Two of these nuclei contain the majority of serotonergic neurons and they send long projections throughout the whole brain, releasing serotonin. Remarkably, the number of serotonergic neurons is very small compared to the total number of brain cells, but they are involved in many brain important functions – from basic regulation of sleep, feeding and body temperature, to involvement in mood and psychiatric disorders.

The serotonin system is well known as the target for antidepressant drugs (eg. SSRIs). These drugs inhibit the reuptake of serotonin after its release by serotonergic neurons and thereby boost the overall levels of serotonin in the brain. Unfortunately, these drugs do not work well in all patients and have substantial side effects. Similarly, decades of experimental research into the function of serotonin has revealed conflicting results and no clear role of this neuromodulator. A possible reason for the discrepancy in results, of both the pharmacological therapies and the experiments, may be that targeting all serotonergic neurons as a single population is not useful due to the fact that the neurons are a heterogeneous population and play roles in different types of functions.

Due to technological advancements, it is becoming possible to divide the general group of serotonin neurons into subpopulations. For example, instead of defining serotonergic neurons solely based on the fact that they release serotonin, it is now also possible to subdivide them based on other characteristics. For example, different groups of neurons express different genes, or contain different electrical properties which affects their communication with other neurons. The aim of this thesis was to study the differences in anatomical connectivity of serotonergic neurons which reside in the two main nuclei – the dorsal and median raphe nuclei. All neurons are part of a complex wiring diagram in the brain, they receive input signals from and send

output signals to other neurons in the brain. The activity of neurons is regulated by the input signals they receive.

In order to map the connections of serotonergic neurons, we used a technique that combines genetics and viruses. The technique takes advantage of rabies, a naturally occurring virus that likes to infect neurons and to spread in a retrograde direction, specifically infecting the neurons that directly contact the originally infected cell, ie. their direct 'inputs'. Using genetic engineering, we modified this normal rabies virus, so that we can specifically infect only serotonergic neurons in the mouse brain, while leaving other types of neurons unaffected. In addition, the virus was modified so that it cannot indefinitely spread from neuron to neuron, as it normally does, instead its spread is limited to only one connection between neurons, staying stuck in the cells that directly connect to serotonergic cells. Using microscopy, we were then able to visualise both the serotonergic neurons and their direct inputs throughout the brain.

In paper I, we identified a large number of input neurons arising in different parts of the brain, that directly connect to serotonergic neurons. In other words, we for the first time identified which brain regions and specific cells directly regulate the activity of serotonergic neurons. The resulting atlas describing tens of thousands of individual connections will act as a blueprint for future studies to deconstruct and reveal the function of these connections. In paper II, we took a closer look at the wiring structure of the serotonergic circuit. In addition to mapping the inputs of serotonergic neurons, we also took into account their outputs. Two specific output regions were selected, one to the prefrontal cortex and another to the striatum regions in the forebrain. Both of these regions may play a role in regulating states such as mood and motivation, and both receive substantial amounts of serotonin release from the dorsal raphe nucleus. We wanted to test whether the serotonergic neurons that project to these two regions receive separate or overlapping inputs. The results mainly showed overlapping sets of inputs, with the exception of a few regions that preferred communicating with one population of neurons over the other. In the future, it will be interesting to determine what effect this connectivity difference makes on specific behaviors. In paper III, we studied how specific cell types in the striatum region communicate with each other, and showed that there are specific rules for the communication in this brain region. Combining the rules of this type of local communication within one brain area, and long–range communication between different brain areas should ultimately lead to a more complete understanding of how the brain works.

In summary, the work of this thesis mapped and defined the types of neural connections that involve the serotonergic neurons of the brain. In future research, it will be important to continue to define subtypes of serotonergic neurons, be it based on connectivity, as in this thesis, or on molecular and genetic features - or even on the combination of both. New findings should ultimately lead to a better understanding of brain function and development of more precise therapies for devastating neuropsychiatric disorders.

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