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MOLECULAR AND FUNCTIONAL NEUROANATOMY OF MOOD DISORDER CIRCUITS

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Molecular and functional neuroanatomy of mood
disorder circuits
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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We have worked all our lives. I've been a seamstress, I've been a kindergarten teacher, I've been a cook. Being able to give something to my children now and seeing them enjoy it, is the most precious thing in life.

Anny Robes, Grandmother Supreme, 2015

Strive not to be a success, but rather to be of value.

Albert Einstein

To my mother,
My father,
And all my Annys

ABSTRACT

The ability to interpret and react to external stimuli is essential for survival. An organism has to be able to predict a favorable outcome, and, more importantly, a non-favorable one that could mean danger for life.

The mammalian brain has developed a number of strategies to interpret external stimuli, to learn the value of a stimulus and to make decisions that promote wellbeing. The underlying neuronal networks are intricate and require numerous interactions in order to provide adequate flow of information. Consequently, slight imbalances in these networks can have disastrous results. In humans, for example, imbalances in monoaminergic neurotransmission can lead to the development of affective disorders like depression and anxiety or brain disorders like schizophrenia, Parkinson's disease or addiction. The neurons involved in these mechanisms are being researched extensively, using a plethora of state of the art methods that examine neuronal activity, connectivity and precise action during behavior, just to name a few. The above-mentioned diseases are fairly common in the global population, a fact that shows how crucial it is to understand the underlying mechanisms and to develop adequate treatment.

The aim of this thesis is to describe the molecular and neuroanatomical properties of neural circuits involved in reward and punishment prediction and decision-making. In chapter 1, I will describe the monoaminergic systems in the mouse brain, mainly focusing on the serotonergic and dopaminergic systems and their involvement in behavior and mood disorders. Chapter 2 reviews classical and current literature on the neural circuits that regulate reward and aversion, with the main focus on the basal ganglia, the habenular complex and the lateral hypothalamic area. Chapter 3 will describe the methodology used to research these circuits on which this thesis is based. More specifically, I will focus on the emergence of big scale single-cell (and single-nuclei) RNA sequencing and in situ hybridization techniques and data analysis.

In **paper 1**, we examined the role of the lateral habenula in aversive behavior and the underlying network connections. A main focus lies on the glutamatergic projections from the lateral hypothalamic area that regulate lateral-habenula activity in fear and avoidance behavior. Molecular distinctions between glutamatergic neurons from neighboring Globus pallidus interna and lateral hypothalamus facilitate the identification of input to the LHb in aversive behavioral tasks.

In **paper 2**, we identified spatial, striosomal and cell-type specific molecular properties of the mouse striatum. We were able to show molecular distinctions between patch, exopatch and matrix SPNs in the striatum. In addition, we reveal a

new spatial map of the striatum and identified a unique SPN type that is independent of this aforementioned spatial code, direct or indirect pathway, or patch/exopatch/matrix characterizations.

In **paper 3**, we show a molecular map of the entire span of the mouse brain with specific emphasis on the spatial distribution of gene expression. This map combines RNA-sequencing with spatial information using spatial transcriptomics.

In **paper 4**, we used a novel two-virus retrograde tracing approach that enabled us to tag the nuclei of neurons sending monosynaptic projections. This allowed us to show the molecular composition of habenula and lateral hypothalamus neurons that directly target serotonergic neurons in the dorsal raphe nucleus and dopaminergic neurons in the ventral tegmental area.

To conclude, genetic targeting of specific neurons is a valuable tool to unravel the exact function of a network. The studies included in this thesis provide new insight into the molecular and neuroanatomical properties of brain circuits involved in behavior. This allows for the identification of possible new genetic targets for behavioral research and ultimately, better understanding of underlying mechanisms.

LIST OF SCIENTIFIC PAPERS

- I. A hypothalamus-habenula circuit controls aversion
Lazaridis I., Tzortzi O., Weglage M., **Märtin A.**, Xuan Y., Parent M., Johansson Y., Fuzik J., Fenno L.E., Ramakrishnan C., Silberberg G., Deisseroth K., Carlén M., Meletis K.
Molecular Psychiatry. 2019 Sep;24(9):1352-1368
- II. A spatiomolecular map of the mouse striatum
Märtin A., Calvigioni D., Tzortzi O., Fuzik J., Wärnberg E., Meletis K.
Cell Reports. 2019 Dec 24; 29(13):4320-4333.e.5.
- III. Molecular Atlas of the Adult Mouse Brain
Cortiz C., Navarro J.F., Jurek A., **Märtin A.**, Lundeberg J., Meletis K.
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- IV. Molecular diversity of Habenula and Lateral Hypothalamus neurons projecting to serotonergic neurons in the dorsal raphe and dopaminergic neurons in the ventral tegmental area.
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Nature Neuroscience. 2018 Jan;21(1):139-149.

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LIST OF ABBREVIATIONS

5HT	serotonin	mAChRs	muscarinic acetylcholine receptors
Adora2a	adenosine receptor 2a	MDD	major depressive disorder
BG	Basal Ganglia	MHb	medial habenula
cDNA	copy DNA	MOA	monoamine oxidase
CS	conditioned stimulus	MRN	median raphe nucleus
D1	Dopamine receptor 1	MSNs	medium spiny neurons
D2	Dopamine receptor 2	NA	Noradrenaline
DA	dopamine	NAc	nucleus accumbens
DAT	Dopamine transporter	nAChRs	nicotinic acetylcholine receptors
DBS	deeb brain simulation	OCD	obsessive-compulsive disorder
DDC	DOPA decarboxylase	Oprm1	μ -opioid receptor
DE	differentially expressed	Pdyn	Prodynorphin
DRN	dorsal raphe nucleus	Penk	Proenkephalin
DS	dorsal striatum	PFC	prefrontal cortex
eSPNs	eccentric spiny projection neurons	QC	quality control
FACS	Fluorescence activated cell sorting	RMTg	Rostromedial tegmental nucleus
FISH	fluorescent in situ hybridization	SCS	single cell sequencing
fMRI	functional magnetic resonance imaging	sm-FISH	single molecule fluorescent in situ hybridization
GABA	Gamma Aminobutyric Acid	SN	substantia nigra
GFP	Green Fluorescent protein	SNc	substantia nigra pars compacta
GPe	Globus Pallidus externa	SNr	substantia nigra pars reticulate
GPI	Globus Pallidus interna	snRNA-seq	single nuclei RNA sequencing
HbX	Novel HbX area with intermingled MHb and LHb neurons	SSRI	selective serotonin reuptake inhibitors
HC	habenular complex	STN	subthalamic nuclei
Htr2c	serotonin receptor 2c	Tac1	Tachykinin Precursor 1
Htr5b	serotonin receptor 5b	TPH2	tyroxine hydroxylase 2
IHC	immunohistochemistry	tSNE	t-Stochastic Neighbor Embedding
IPN	interpeduncular nucleus	TSO	template switch oligo
LCM	laser capture microdissection	US	unconditioned stimulus
LH	lateral hypothalamic area	V1	visual cortex
LHb	Lateral habenula	VS	ventral striatum
LSD	lysergic acid diethylamide	VTA	ventral tegmental area

CHAPTER 1

1 MONOAMINERGIC ACTION IN THE BRAIN – FOCUS ON SEROTONIN AND DOPAMINE

1.1 INTRODUCTION

There are three neurotransmitters that are commonly referred to as the major monoamines in the brain: serotonin, dopamine and noradrenalin. Each of these neurotransmitters is associated with specific functions. Serotonin is involved in the regulation of mood (Cools et al., 2008), sleep (Monti, 2011) and appetitive behavior (Strasser et al., 2016). Dopamine plays a major role in movement, motivation and reward (Cools, 2008), while noradrenaline is implicated in the regulation of attention, learning and memory processes (Hensler et al., 2013). While the many functions of noradrenalin are certainly fascinating in their own right, this thesis will focus on serotonin and dopamine actions.

1.2 SEROTONIN – A VERY SHORT AND INCOMPLETE HISTORY

In 1937, the hormone ‘enteramine’ was discovered in the enterochromaffin cells of the gut by Vialli and Erspamer, and it was later coined with the charming name 5-hydroxytryptamine (5HT) (ERSPAMER & ASERO, 1952) or serotonin (for “serum tonic”). It was soon detected in the brain of dogs (AMIN et al., 1954) and rabbits and rats (BOGDANSKI et al., 1956) and was hypothesized very early on to be a “neurohumoral” agent, or neurotransmitter. Interestingly, Gaddum et al, as early as 1953 suspected that serotonin likely plays a role in mood regulation after self-administration (!) of lysergic acid diethylamide (LSD) and subsequent reports that it works by supressing serotonin function in the periphery (GADDUM, 1953). Technical advancements in the 1960s allowed for the identification and visualisation of 5HT-positive neurons (and DA- and NA-positive neurons) in the rat midbrain region (Annica Dahlström & Fuxe, 1964). Since then research on the neurotransmitter serotonin has tried to build a complete picture of its many mechanisms and exact functions in behaviour and continues to do so.

1.3 SEROTONIN FUNCTION IN THE CNS

Serotonergic neurons in the raphe nuclei are the source of 5-HT in the brain. There it is synthesized from the amino acid tryptophan to serotonin by enzymes tyrosine hydroxylase 2 (TPH2) and DOPA decarboxylase (DDC). Upon activation, serotonin is released via synapses or more diffusely from axons and dendrites (De-Miguel & Trueta, 2005; Fuxe et al., 2010). Its action is terminated through reuptake by the serotonin transporter in the presynaptic neuron.

Serotonergic neurons in the brain are sparse and mainly located in the hindbrain. However, tracing studies have shown that the relatively low number of neurons project to most areas in the mammalian brain (Jacobs & Azmitia, 1992) and receive projections from a large number of areas as well (Pollak Dorocic et al., 2014). Recent studies have revealed a large heterogeneity between the serotonergic centers of DRN (the dorsal raphe nucleus) and the MRN (median raphe nucleus) on a connectivity, electrophysiological, developmental and functional level (Fernandez et al., 2016; Okaty et al., 2019; Ren et al., 2018; Teissier et al., 2015). In addition, studies have reported electrophysiological differences between serotonergic cells within the MRN (Okaty et al., 2015) and suggested distinct functions in behavior (Kim et al., 2009). Other studies report evidence for subpopulations of DRN serotonergic neurons based on projection target and associated anatomical and physiological characteristics (Ren et al., 2018). Optogenetic manipulations of serotonergic neurons in the DRN suggest diverse behavioral functions of proposed physiological subpopulations of DRN 5HT neurons (Cohen et al., 2015). Finally, single-cell RNA sequencing of 5HT neurons in DRN and MRN confirm molecularly distinct types of 5HT neurons based on developmental and anatomical origin (Okaty et al., 2015; Ren et al., 2019). In addition to the great diversity of serotonergic neurons, there are 14 known serotonin receptors expressed in the brain which reflects its functional variability (Palacios, 2016). These receptors are subdivided into inhibitory (5-HT₁ and 5-HT₅) and excitatory types (5-HT₂, 5-HT₃, 5-HT₄, 5-HT₆ and 5-HT₇,) and all but one are G-protein coupled receptors. Only the 5-HT₃ receptor types are ligand-activated ion channels (Reeves & Lummis, 2002). There is a great amount of research done on the function of these receptors. A review of all this information would be beyond the scope of this thesis.

1.4 SEROTONIN AND MOOD DISORDERS

Serotonin has become the focus of research into depression in the late 1960s, when the “serotonin hypothesis of depression” was postulated (Coppen, 1969). According to this hypothesis, an increase in free synaptic serotonin might have antidepressant effects. This seemed to be confirmed by the therapeutic effects of selective serotonin reuptake inhibitors (SSRIs) in depressive patients. These therapeutics

hypothetically increase the duration of serotonergic transmission in the synapse and have become the first choice of treatment for depression, but also anxiety and panic disorders, appetitive disorders and obsessive-compulsive disorder (OCD) (Baumgarten & Grozdanovic, 1998). The effectiveness of SSRIs in these disorders has supported the hypothesis that decreased serotonin transmission facilitates the development of depression in vulnerable individuals. However, about one third of patients do not respond to SSRI treatment (Fava & Davidson, 1996) and the effect of SSRIs is not immediate but shows a therapeutic delay of a few weeks (Charney et al., 1982). In addition to SSRIs other drugs have emerged that target the serotonergic system. One example is vortioxetine that activates some serotonin receptors and inhibits others with marked anxiolytic and antidepressant effects (Sanchez et al., 2015). This complexity shows the need for research into the different mechanisms serotonin has on different receptors in different brain areas which is, as of now, poorly understood. To make matters more complicated, it has been reported that DRN and MRN have distinct effects on depression and anxiety (Lechin et al., 2006). Activation of the MRN caused an increase in anxiety behavior in mice (Ohmura et al., 2014) while DRN inhibition was associated with depressive behavior (Teissier et al., 2015). Another, more recent hypothesis postulates a “high serotonin hypothesis of depression”, rather than the classic hypothesis described above. This hypothesis incorporates conflicting results of numerous studies on the effect of serotonin transmission on depressive states and argues that not only serotonin transmission is vital, but also the context in which it is looked at. This “state” of the whole organism is thought to influence serotonin's effects (Andrews et al., 2015).

To summarize, serotonin's importance in mood disorders like depression and anxiety is undisputed but its role is not well understood despite the large research effort. We have to accept the fact that not one compound causes one disease. This simplistic view, while attractive, has been disproved time and time again. Depression in itself is a complicated disorder with a variety of symptoms, so it is not far-fetched that its causes are complicated as well. Or is it the other way around?

1.5 DOPAMINE – A VERY SHORT AND INCOMPLETE HISTORY

Dopamine received attention as its own compound in the 1950, after it was considered a precursor of noradrenaline for a good 40 years (Hornykiewicz, 2002). Research on monoamine oxidase (MOA, the enzyme that breaks down monoamines) in the 1950s suggested an important physiological role of dopamine when its capability to reduce blood pressure was discovered, and that MOA increased that effect (Blaschko & Hope, 1957). A few years later high levels of dopamine were discovered in the striatum (Bertler & Rosengren, 1959). It was this finding that inspired researchers to look at dopamine in Parkinson patients and they confirmed a loss of dopamine in post-mortem brains of Parkinson patients

(EHRINGER & HORNYKIEWICZ, 1960). Most significantly, one year later L-Dopa was first tested as a therapy for Parkinson's disease (BIRKMAYER & HORNYKIEWICZ, 1961). Immunohistochemical examination later confirmed dopamine transmission between the ventral tegmental area (VTA) and the striatum (Dahlström & Fuxe, 1965). It was established as neurotransmitter with the discovery of dopamine receptors in the early 1980s (Marsden, 2006).

1.6 DOPAMINE FUNCTION IN THE CNS

Dopamine as a neurotransmitter is involved in a number of processes. It is synthesized by dopaminergic neurons located in the VTA and the substantia nigra pars compacta (SNc). The VTA sends dopaminergic projections to the nucleus accumbens (NAc) in ventral striatum while the SNc targets the dorsal striatum via a nigrostriatal pathway. In addition, these structures send projections to limbic areas such as amygdala, to cortical structures, thalamus and hypothalamus (R M Beckstead, 1979; Robert M. Beckstead et al., 1979). In return, these structures receive massive input from a variety of regions (Watabe-Uchida et al., 2012).

Historically, dopamine is first and foremost involved in regulating movement. The classical model of movement involves the so-called direct and indirect pathways in striatum that are driven by activation of dopamine receptors D1 and D2, respectively. Indeed, optogenetic activation of D1 in striatal MSNs causes inhibition of the main output nucleus substantia nigra pars reticulata (SNr), thereby facilitating movement. Activation of D2 shows opposite effects, activating the SNr and thereby inhibiting movement (Kravitz et al., 2010). Disturbances of dopamine signaling are associated with serious movement disorders like Huntington's disease (Cepeda et al., 2014), Tourette's syndrome (Maia & Conceição, 2018) and the aforementioned Parkinson's disease. In addition to control of movement, dopamine is involved in motivation and learning, positive and negative reinforcement, and decision-making (Atkinson-Clement et al., 2019; Petzold et al., 2019). Electrophysiological experiments in primates showed phasic activation of VTA and SNc dopamine neurons in response to (expected and unexpected) rewarding cues and the opposite could be detected during unexpected reward omission (Schultz, 1997). This activity has since been supported in fMRI studies in humans (D'Ardenne et al., 2008) and optogenetic activation of DA neurons in VTA and SNc in mice (Cohen et al., 2012). Consequently, optogenetic inhibition of these DA neurons was associated with a negative prediction error that could extinct a previously learned positive value (Hamid et al., 2015). Other studies have reported excitation of dopaminergic neurons in ventral VTA at the onset of a footshock (Brischoux et al., 2009) while they are inhibited in other parts of the VTA by stimuli of the same aversive value (Ungless et al., 2004). Taken together, these findings suggest a large functional diversity of dopaminergic signaling.

1.7 DOPAMINE AND MOOD DISORDERS

Dopamine is not only implicated in the etiology of movement disorders (see above) but is also associated with other brain disorders. Anhedonia, the inability to experience pleasure, is one often-described symptom of Parkinson's disease (Zahodne et al., 2012), schizophrenia (Strauss & Gold, 2012), relapsing substance abusers (Volkow et al., 2002) and major depression (MDD) (Vrieze et al., 2014). All of these disorders have been associated with dysfunctions in dopamine signaling as has anhedonia (Der-Avakian & Markou, 2012). Studies in human depressive patients showed a decreased dopamine transporter (DAT) binding hinting at a possible decrease in DA concentrations (Sarchiapone et al., 2006). Animal studies showed decreased DA transmission and decreased striatal dopamine activity in depression models and depression-related behavioral tasks (Kram et al., 2002; Pruessner et al., 2004). In addition, inhibition of VTA DA neurons was linked to depressive-like behavior in mice (Tye et al., 2013).

1.8 MONOAMINERGIC INTERACTIONS

The two monoamines discussed in this thesis show a number of similar functions. Therefore, it is not surprising that there are numerous studies reporting interactions between the two systems. In 1965, initial studies reported serotonergic nerve terminals in the VTA and substantia nigra (SN) (Fuxe, 1965) and it was proven that 5HT neurons from DRN send projections to both areas (Steinbusch et al., 1981) while MRN only targets VTA (Azmitia & Segal, 1978). More recent tracing studies showed that VTA sends dopaminergic projections to the DRN and that these projection neurons in turn receive inputs specifically from the lateral hypothalamic area (LH). Additionally, the same study showed that this VTA-DRN connection is reciprocal, without definitively showing if the projection from DRN is serotonergic or not (Pollak Dorocic et al., 2014).

A functional study observed increased serotonin and reduced dopamine transmission in the nucleus accumbens during depressive states (Bland et al., 2003). This seems to confirm an early hypothesis that serotonin inhibits dopamine transmission in the brain (Samanin & Garattini, 1975). Whether this inhibiting function is a direct transmission, thus via certain 5HT receptors, or acts via GABAergic interneurons, is still matter of debate and research (Esposito et al., 2008).

As expected, the more research is done on neural circuits, the more complicated the picture gets. It seems to be clear that there is some kind of interaction between these monoaminergic systems and that there is possible direct connection between dopaminergic and serotonergic neurons. In addition, the effects are mediated by a number of receptors for both neurotransmitters, again indicating a highly intricate functional network.

CHAPTER 2

2 BASAL GANGLIA

2.1 INTRODUCTION

The basal ganglia (BG) are subcortical nuclei consisting of the striatum, globus pallidus, substantia nigra and the subthalamic nucleus. These highly conserved structures form a network that is involved in movement control, learning and memory and behavioral regulation. Input to the basal ganglia originate from cortex, the limbic system and thalamus (Redgrave et al., 2010). The basal ganglia are heavily implicated in pathophysiology of Parkinson's disease and have been the focus of research into depression. This thesis will give an overview of the key players in the basal ganglia circuits and their function.

2.2 STRIATUM

The striatum is classically seen as the entryway to the basal ganglia. It receives mainly glutamatergic input from several cortical areas and thalamus, and is involved in the regulation of movement, motivation and evaluation of external stimuli (Costa et al., 2016; Samejima et al., 2005). Cortical input comes from areas associated with limbic, cognitive and sensorimotor functions (Nambu et al., 2002). The striatal target areas of these cortical projections are topographically distinct, with limbic projections targeting ventromedial parts, sensorimotor projections being directed at dorsolateral striatum and cognitive cortical input targeting the region in between these two (Wiesendanger et al., 2004). These functionality-based pathways are the first part of parallel cortico-basal closed loops. Essentially, the different cortical areas send information to the different parts of striatum and receive feedback from these areas via globus pallidus, substantia nigra and thalamus. The striatum as the basal ganglia entry therefore has an integrating function that directs environmental information to downstream areas to initiate an appropriate response (Alexander et al., 1986; McHaffie et al., 2005). The striatum is a relatively large area, which is nevertheless considered as one functional entity. However, based on histological, functional, connectivity and molecular evidence, the striatum can be subdivided further. The most basic subdivision is into dorsal (DS) and ventral parts (VS). These compartments are histologically and functionally different. The VS with the Nucleus accumbens (NAc), for example, receives dopaminergic input from VTA and is mainly involved in reward- and emotion, goal-directed behavior and motivation (mainly the NAc). It is also the

part of striatum that receives input from amygdala and hippocampus (Fudge et al., 2002). The DS on the other hand, receives dopaminergic input from SNc and is associated with motor control, action selection and initiation (Balleine et al., 2007).

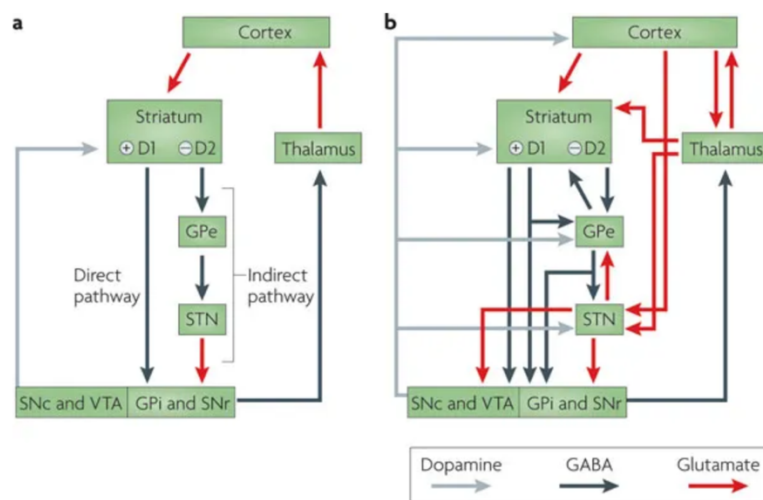


Figure 1: Organization of intrinsic connections within the basal ganglia (a) Model based on the influential proposal by Albin and colleagues³, according to which the output of the basal ganglia is determined by the balance between the direct pathway — which involves direct striatonigral inhibitory connections that promote behaviour — and the indirect pathway — which involves relays in the external globus

pallidus (GPe) and subthalamic nucleus (STN), and suppresses behaviour. The balance between these two projections was thought to be regulated by afferent dopaminergic signals from the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) acting on differentially distributed D1 and D2 dopamine receptors. (b) Recent anatomical investigations have revealed a rather more complex organization in which the transformations that are applied to the inputs to generate outputs are less easy to predict. GPe, globus pallidus pars externa; GPi, globus pallidus pars interna; SNr, substantia nigra pars reticulata. Image from (Redgrave et al., 2010)

The vast majority of cells in the striatum are GABAergic neurons called medium spiny neurons (MSNs) that can be divided into two major groups based on their molecular profile and functionality. The classical model of striatal regulation of movement involves the so-called direct and indirect pathways. One group of MSNs expresses the dopamine receptor D1 and constitute the direct pathway. The second group of MSNs express the dopamine receptor D2 and constitute the indirect pathway.

D1+ MSNs of the direct pathway project to the Globus Pallidus interna (GPi) and SNr, which leads to a disinhibition of thalamic projections to the cortex and promotes movement. The D2+ MSNs of the indirect pathway instead project to the Globus Pallidus externa (GPe), which through inhibition of the STN ultimately leads to an inhibition of thalamic projections to cortical areas, thereby inhibiting movement (Kreitzer & Malenka, 2008). While this model is relatively old (from the late 1980s, see Redgrave et al, 2010), it is still valid, albeit more complicated than initially proclaimed (Fig.1 a vs b).

Another topographical striatal division is into dorsolateral and dorsomedial parts of striatum. The dorsolateral striatum is commonly associated with sensorimotor functions while the dorsomedial striatum is associated with cognitive functions

(Redgrave et al., 2010). Lesion studies in rodents have shown that inactivation of the dorsomedial striatum resulted in diminished learning in an action-outcome paradigm while dorsolateral striatum lesions resulted in a disruption of habit-formation (Yin et al., 2004, 2006).

The patch and matrix structures in the striatum form another anatomical and functional subdivision of the striatum. The patch compartment is an interconnected three-dimensional structure that spans the striatum and is classically defined by the expression of the μ -opioid receptor (*Oprm1*) and dopamine receptor D1 (Gerfen, 1984). Striatal patches project to the SNc and have been linked to value learning through these projections (Ragsdale & Graybiel, 1988). The patches receive limbic input and are strongly associated with emotional signaling (Gerfen, n.d.). In contrast, the matrix compartment does not express *Oprm1* and is composed of D1 and D2 positive MSNs. Matrix neurons receive inputs from the SNc, suggesting a possible indirect interaction with patch signaling, as there appears to be no direct communication between the compartments (Banghart et al., 2015; Eblen & Graybiel, 1995).

2.2.1 CELL TYPES

The described MSN types from the direct and indirect pathways differ from each other on a molecular scale. Aside from the dopamine receptors D1 and D2 in direct and indirect pathway MSNs, respectively, expression of a number of other genes characterize the two MSN types. The D1+ MSNs highly express *Tac1* (substance P) and *Pdyn* (Prodynorphin) while the D2+ MSNs express *Adora2a* (adenosine receptor 2a) and *Penk* (Proenkephalin).

A large-scale transcriptomics study on mouse brain cells revealed additional molecular properties of striatal neurons. A new striatal cell type was identified with molecular properties that overlap with indirect and direct MSNs (expression of *Drd1a* and *Adora2a*) and did not belong to the interneuron cell types and patch or matrix compartments. The distribution of these *Otof*-expressing cells called eSPNs (for eccentric spiny projection neurons) did not show any spatial preference (Saunders et al., 2018). An earlier study used single-cell RNA sequencing methods and described subpopulations of direct and indirect MSNs based on their transcriptome profile, with a small population expressing both dopamine receptors (D1 and D2) that usually separate the direct and indirect MSNs. Interestingly, this study also observed gene expression gradients in their MSN populations (Gokce et al., 2016). These results are highly relevant for further research into the functional domains of the striatum.

Another cell type found in the striatum are the interneurons. These are GABAergic or cholinergic and are typically distributed over the entire striatal area, with the cholinergic interneurons residing in the matrix compartment in patch-matrix

border regions (Brimblecombe & Cragg, 2017).

They innervate and modulate striatal MSNs and are relatively diverse. The GABAergic interneurons can be subdivided into *Pthlh+*, *Sst/Npy+*, *Th+*, *Cck/Vip+*, *Cck+* and *Npy+* interneurons, with the fast-spiking *Pvalb/Pthlh+* interneurons forming the largest population (Muñoz-Manchado et al., 2018).

2.3 GLOBUS PALLIDUS

The globus pallidus (GP) is a downstream structure in the basal ganglia circuits and consists of an internal (GPi) and an external segment (GPe). The GP is an important output nucleus from the basal ganglia and it is involved in both the direct and indirect pathways of movement regulation (Gerfen, 2004). Both subparts of the GP receive glutamatergic input from the Subthalamic Nucleus (STN) and GABAergic projections from striatum.

2.3.1 THE GPE

The GPe forms the center of the basal ganglia circuits due to its input and output structure. It mainly receives input from the striatum and from the STN and sparse projections from cortex, thalamus, GPi and SNc ((Parent & Hazrati, 1995; Yasukawa et al., 2004). The vast majority of GPe neurons are GABAergic and these send projections to striatum, GPi, SNr and STN (H. Kita, 2007; T. Kita & Kita, 2011; Shink & Smith, 1995). The GABAergic projection neurons from GPe can be subdivided into the so-called arkypallidal neurons and the prototypic cells. The latter population is characterized by expression of *Pvalb* (Parvalbumin) and targets GPi, SNr and STN while the former type lacks *Pvalb* expression and instead contains *Penk* (Proenkephalin) expressing cells that exclusively targets striatum (Mallet et al., 2012). The GPe is mainly associated with the indirect pathway (H. Kita, 2007).

2.3.2 THE GPI

In contrast to the GPe, the GPi is comprised of GABAergic and glutamatergic neurons and, together with the SNc, forms the main output center of the basal ganglia. A special feature of the GPi projection neurons is that those neurons targeting habenula are co-releasing GABA and glutamate (Shabel et al., 2014). In addition, the GPi sends projections to thalamus, cortex, striatum and STN (H. Kita, 2007; T. Kita & Kita, 2011). As the main output structure, the GPi is involved in both the direct and indirect pathways.

2.4 SUBSTANTIA NIGRA AND SUBTHALAMIC NUCLEUS – IN BRIEF

The substantia nigra is a dopaminergic structure in the midbrain and is subdivided into the pars compacta (SNc) and the pars reticulata (SNr). The latter is the main output nucleus of the basal ganglia and sends GABAergic projections to thalamus, cortex and brainstem (Deniau et al., 2007).

The STN is a highly glutamatergic structure and is involved in the hyperdirect and the indirect pathway of the basal ganglia. It receives glutamatergic input from cortical regions and GABAergic projections from GPe. It is involved in motor, cognitive and limbic functions (Teagarden & Rebec, 2007) and projects to GPe, GPi and SNr (T. P. Ma & Geyer, 2018).

2.5 PAPER II – A SPATIOMOLECULAR MAP OF THE MOUSE STRIATUM

2.5.1 AIMS

The goal of this study was to identify molecular subpopulations in striatal SPNs and identify distinctive molecular markers for the separation of patch and matrix compartments of the striatum. The standard mouse brain atlases currently used divide the large area of the striatum into DS and VS (as described earlier), which is limited if the connectivity pattern of cortical input to and BG output from the striatum are taken into account. Our attempt in this study was to add molecular information to the existing knowledge and to try and give more dimension to the subdivision of this important area.

In order to accomplish our goals, we bred three reporter mouse lines, *Oprm1*-Cre:TdTomato, *Oprm1*-Cre:H2BG and *Vgat*-Cre:H2BG mice. Animals with the “:H2BG” genotype displayed a GFP tag on the histone 2B in the nucleus in a Cre-dependent manner. As histone 2B is incorporated in the chromatin, we used this tag to isolate nuclei of *Oprm1*⁺ and *Vgat*⁺ cells from the DS and performed snRNA sequencing on the whole population. In order to obtain single GFP⁺ nuclei, we performed a simple nuclear isolation protocol, followed by Fluorescence-Activated cell sorting (FACS) that sorted individual nuclei based on their GFP emission profile. Isolated nuclei were sequenced and the resulting gene expression matrix was used for analysis.

We used the *Oprm1*-Cre:TdTomato⁺ mouse line to characterize the morphology and the spatial distribution of the *Oprm1*⁺ neurons in striatum and to identify patch vs matrix compartments and exopatch neurons. In addition, we used this same mouse line for a number of single molecule fluorescent in situ hybridization (sm-FISH) experiments to show the validity of our identified genetic markers. Whole brain TdTomato report gene expression in the transgenic mouse line was

done using tissue clearing methods in combination with 3D imaging and immunohistochemical experiments (IHC).

2.5.2 RESULTS AND CONCLUSIONS

We reported the three dimensional anatomy of striatal patch and matrix compartments in the TdTomato+ mouse line. Importantly, we demonstrated the distribution of striatal *Oprm1*+ in exopatch cells. Analysis of the acquired images showed that roughly 90% of *Oprm1*+ neurons belonged to the patches while the rest formed the exopatch population.

One of our main interests in this study was to identify markers that separate patch and matrix compartments. We therefore combined snRNA-seq data from *Vgat*+ and *Oprm1*+ (i.e. patch) neurons. Unbiased analysis of the pooled dataset revealed separate clusters and subsequent identification of cell origin in the resulting clusters enabled us to distinguish between known patch nuclei and the *Vgat*+ SPNs. We observed enrichment of the marker *Sema5b* in the patch nuclei versus enrichment of *Id4* in non-patch nuclei, and we confirmed a patch/matrix differentiation by these markers using smFISH experiments. Interestingly, these two markers were also useful in the differentiation between patch and exopatch cells. While both populations expressed *Oprm1*, the patch cells were *Sema5b*-positive and the exopatch cells expressed *Id4*.

Our next goal was to examine if there are molecular markers for distinct spatial domains that were independent of cell-type and patch/matrix across the entire SPN population (see Fig. 2C). Indeed, clustering analysis showed expression gradients of markers that displayed a spatial pattern within all clusters. The three genes *Dlk1*, *Crym* and *Gpr155* did not mark single clusters but followed spatial gradients within the clusters of cell types. Using smFISH, we demonstrated high expression of *Crym* in the medial DS, a high expression of *Dlk1* in the ventromedial part and high levels of *Gpr155* in the lateral part of DS.

Unexpectedly, we identified a potential new SPN subtype. Our clustering results included one particular small cluster that piqued our interest, which was positive for D1 and D2. Moreover, this cluster did not belong to the identified interneuron clusters, nor to patch or matrix and neither did it display a spatial distribution. Differential expression analysis revealed that the gene *Col11a1* marked this cell population. Expecting it to be of the eSPN type (*Otof*+/*Pcdh8*+, Saunders et al, 2018), we saw that this cluster includes *Otof*+/*Pcdh8*+ cells but the markers were not specific for this cell cluster. We detected very high *Otof* expression in *Sst/Npy* interneurons and high *Pcdh8* expression in *Pthlh* interneurons. In addition to the distinct molecular characteristics, injection of retrobeads into the SN revealed that the *Col11a1*+ cell type formed striatonigral projections.

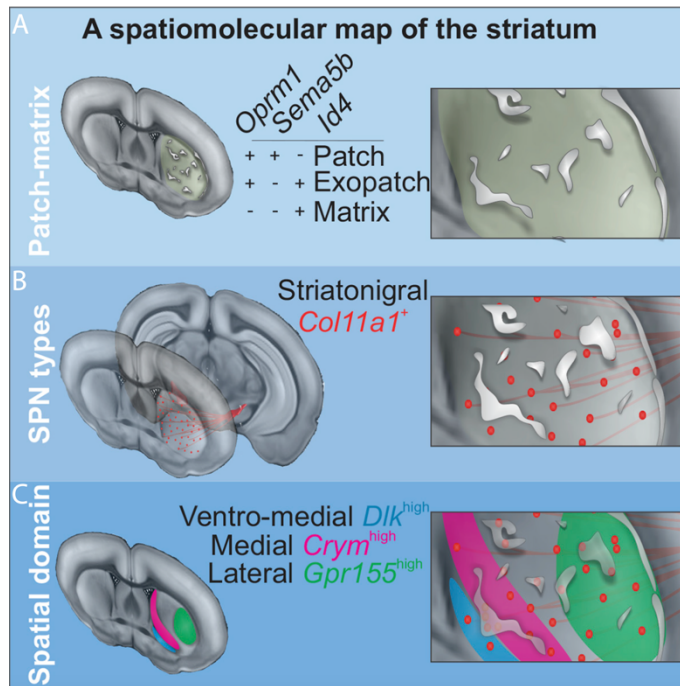


Fig. 2
 (A) The three molecular markers *Oprm1*, *Sema5b* and *Id4* distinguishing patch/exopatch/matrix compartments in striatum. (B) Distribution of *Col11a1* striatonigral projection neuron type throughout the striatal area. (C) Spatial compartments in the striatum, marked by *Dlk1*-, *Crym*- and *Gpr155*- expression. (Image from (from Märtin, A. 2019, Cell Reports, 29(13), 4320–4333.e5.)

Taken together, these findings demonstrate the molecular diversity of the dorsal striatum. This study increased insight into spatial striatal domains and highlights possible new targets for genetic manipulation and behavioral experiments. Moreover, we provide a starting point for functional analysis of these striatal domains. Likewise, we expanded the list of patch/matrix markers that can aid in researching the functional differences of these striatal compartments in a more directed way. Finally, the identification of a potentially new SPN type is exciting and future research may eventually resolve its functions.

CHAPTER 3

3 THE HABENULA

3.1 INTRODUCTION

The habenular complex (HC) is an epithalamic structure that is highly preserved throughout all vertebrate species (Stephenson-Jones et al., 2012). In mammalian species it is subdivided into the medial habenula (MHb) and the Lateral habenula (LHb), structures that have very distinct molecular, functional and connectivity properties (Aizawa et al., 2012). This high level of conservation suggests an essential role of the HC for survival of an organism and indeed, it has been associated with roles in motivation and decision-making (Boulos et al., 2017). It is a highly glutamatergic structure that connects the forebrain with mid- and hindbrain structures.

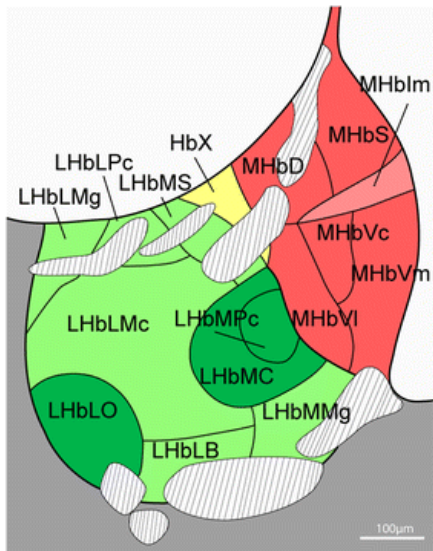


Fig. 3: Schematic representation of habenular subnuclei in the mouse brain. LHb in green, MHb in red, HbX in yellow. LHbMPC (parvocellular subnucleus of the medial division of the LHb), LHbMC (central subnucleus of the medial division of the LHb), and LHbLO (oval subnucleus of the lateral division of the LHb) can be identified by gene localizations as found in the present report and are shown in *dark green*. MHb subnuclei are presented in *red* with the novel MHbIm (intermediate subnucleus of the MHb) area in light red. The HbX area is displayed in *yellow*. Hatched fields represent fiber bundles. MHbD—dorsal subnucleus of the MHb; MHbS—superior subnucleus of the MHb; MHbVl—lateral subnucleus of the ventral MHb; MHbVc—central subnucleus of the ventral MHb; MHbVm—medial subnucleus of the ventral MHb; LHbLMg—marginal subnucleus of the lateral division of the LHb; LHbLPC—parvocellular subnucleus of the lateral division of the LHb;

LHbLMc—magnocellular subnucleus of the lateral division of the LHb; LHbLB—basal subnucleus of the lateral division of the LHb; LHbMS—superior subnucleus of the medial division of the LHb; LHbMMg—marginal subnucleus of the medial division of the LHb. Bar indicates 100 μm (Image and legend from Wagner et al, 2016, Brain Struct Funct, 221 (1), 39-58 Jan 2016).

3.2 ANATOMY AND CONNECTIVITY

The location of the HC is fairly central in the brain and it connects the forebrain to midbrain and hindbrain areas via specific bundle systems. Moreover, it interacts with monoaminergic centers, specifically the serotonergic and dopaminergic systems (Stephenson-Jones et al., 2012). The two main habenular nuclei are divided into subregions with distinct molecular and connectivity characteristics. The habenular anatomy in the mouse has recently been updated by Wagner et al using anatomical and transcriptomics data from Allen Brain Atlas (Wagner et al.,

2016) (see Fig.1). This analysis revealed nine LHb and six MHb subregions with the addition of a presumed transitional area (HbX) that presented with LHb and MHb characteristics. The subregions of both HC nuclei are defined by molecular properties and neural connections. The MHb receives input mainly from the septum (Viswanath et al., 2013), the VTA and midbrain raphe (Herkenham & Nauta, 1977; Phillipson & Pycck, 1982), and locus coeruleus (Gottesfeld, 1983).

The main output structure of the MHb is the interpeduncular nucleus (IPN), which sends projections to a variety of brain regions that include raphe nuclei and VTA (McLaughlin et al., 2017). However, there is evidence for direct innervation of the VTA by the MHb (Cuello et al., 1978; Watabe-Uchida et al., 2012). The dorsal part of the MHb, defined by neurons expressing substance P, has been shown to connect to the lateral subnucleus of the IPN (Hsu et al., 2016) while the acetylcholine expressing neurons from the ventral MHb target the rest of IPN (Harrington et al., 2016; Herkenham, 1979). Connections from and to LHb have been studied more intensively, with reports of projections from Globus Pallidus internal segment (GPi), Lateral Hypothalamic Area (LH), basal ganglia, basal forebrain, ventral pallidum and lateral preoptic area (Yang et al., 2018). However, these inputs are not uniformly distributed over the LHb structure. The medial part of LHb, for example, receives input from limbic regions (Herkenham & Nauta, 1977; Hikosaka, 2010) while the lateral part is highly innervated by basal ganglia structures like GPi (Herkenham & Nauta, 1977). In addition, the LHb receives dopaminergic input from VTA (Sutherland, 1982). The LHb in turn sends projections to VTA where it targets dopaminergic as well as GABAergic neurons to SNc, DRN and MRN (Omelchenko et al., 2009). It also connects to the RMTg (rostromedial tegmental nucleus), a highly GABAergic nucleus that itself is connected to midbrain monoaminergic regions (Hong et al., 2011; Yang et al., 2018)

3.3 FUNCTIONS

Numerous studies on the HC have reported functions in sleep (Valjakka et al., 1998), reward (Matsumoto & Hikosaka, 2007), pain (Q. P. Ma & Han, 1991), and female sexual behavior (Modianos et al., 1974). Early lesion studies in rats showed a delayed avoidance learning compared to controls and an increased exploratory behavior in an open field task (Nielson & McIver, 1966). These results indicated a function of the HC in modulating aversive stimuli.

3.3.1 THE LHB

More recent studies revealed distinct functions of the two main subnuclei of the HC. Monkey studies revealed that the LHb was activated during non-reward trials of a visual saccade task. This activation was accompanied by an inhibition of dopamine neurons in SNc with a slight delay, indicating that LHb activation caused dopamine inhibition (Matsumoto & Hikosaka, 2007). In addition, activation of the LHb projections to medial VTA in mice caused a strong place aversion (Lammel et

al., 2012). Another study added that activation of RMTg projecting LHb neurons caused an aversive response (Stamatakis & Stuber, 2012). Therefore, the inhibition of dopaminergic neurons by LHb can be direct or indirect. Research on behavioral circuits involving LHb also suggested that glutamatergic activation of LHb by GPi is involved in the signaling of negative stimuli and negative reward prediction (Hong & Hikosaka, 2008). This activation seemed to originate in the border region between GPi and LH. Indeed, inactivation of the LH-LHb connection decreased behavioral response to aversive stimuli (Lecca et al., 2017). The LHb has also been investigated in research into depression. The reciprocal connections with the serotonergic system suggest a possible involvement (Pollak Dorocic et al., 2014). Indeed, studies in human depressive patients reported a decreased habenula volume compared to an unaffected control group (Ranft et al., 2010). Additionally, Deep Brain Stimulation (DBS) of the habenula activity has shown to alleviate symptoms of depression in humans (Sartorius et al., 2010). Research in animal models of depression reported hyperactivity of the LHb (Li et al., 2011). Interestingly, it was shown that 5HT has an inhibiting effect on LHb activity (Shabel et al., 2012), indicating a feedback loop. This regulatory system might be susceptible to small imbalances, as an increase in LHb activity would lead to reduced serotonin transmission and thereby to disinhibition of LHb activity itself. This view is supported by the mentioned hyperactivity of LHb in depressive patients and animal models of depression.

3.3.2 EXCURSION: THE LATERAL HYPOTHALAMUS

As indicated by the name, this structure is part of the hypothalamus and is mainly involved in the regulation of feeding and drinking behavior but also reinforcement of positive external stimuli (Stuber & Wise, 2016). It was also reported to disinhibit dopaminergic neurons in the VTA during feeding, leading to increased dopamine signaling (Nieh et al., 2016). In addition, the LH is directly connected to the serotonergic system by projecting to DRN and MRN (Pollak Dorocic et al., 2014), indicating a possible regulation of the 5HT system by LH signalling. A recent study has described the molecular composition of the LH, extensively detailing the transcriptomic properties of GABAergic and glutamatergic populations of the LH (Mickelsen et al., 2019). Finally, as stated earlier, the LH is connected to the LHb and seems to mediate aversive behaviour via activation of this structure.

3.3.3 THE MHB

The MHb has been less investigated than the LHb. However, interest has increased in recent years. It has been shown that MHb plays an important role in stress (Lecourtier et al., 2004; Mathuru & Jesuthasan, 2013), depression (Shumake et al., 2003), memory (Kobayashi et al., 2013), and nicotine withdrawal (Fowler et al., 2011; Frahm et al., 2011; Salas et al., 2009). Especially the role of MHb in nicotine action is interesting. One of the characteristics of the MHb is the high expression of

nicotinic acetylcholine receptors (nAChRs). Subtypes of these have been extensively studied in the context of nicotine action and addiction in general (Franceschini et al., 2002; Glick et al., 2006; Maskos et al., 2005). Research into human MHb has been limited by technological restrictions that do not allow for distinction between the two HC nuclei in vivo (Strotmann et al., 2013, 2014).

3.4 MOLECULAR PROPERTIES

Recent single-cell RNA-sequencing studies on HC impressively demonstrate the differences between the HC nuclei and between LHb and MHb subnuclei. As expected, the transcriptome profiles of MHb and LHb are distinct. Here is a quick summary of the findings described by Hashikawa et al (2019) and Wallace et al (2020). While there have been descriptions of genetic differences between the HC nuclei, these two studies focus on single-cell transcriptomes of the mouse HC and show connection properties as well.

Both transcriptomic studies distinguish the MHb neurons from LHb neurons by their expression of *Tac2* and *Gap43*, respectively. Both nuclei are glutamatergic and express the vesicular glutamate transporter *Vglut2* (*Slc17a6*). Only MHb however, also expresses *Vglut1* (*Slc17a7*). Differences in serotonergic receptor expression was described: While the MHb expresses *Htr5b*, the LHb expressed *Htr2c* in high levels. The MHb also expresses *Chat* (gene for choline acetyltransferase) and *Slc18a3* (gene for vesicular choline transporter), both of which are required for a cell to transmit acetylcholine. This indicates a co-transmission of acetylcholine and glutamate from the MHb, supporting optogenetic and mouse knock-out studies (Ren et al., 2011; Soria-Gómez et al., 2015). One study found molecular distinctions between subnuclei within the MHb and within the LHb (Wallace et al., 2020). Moreover, one study identified four distinct LHb subpopulations (defined by *Chrm3*, *Vgf*, *Gpr151*, and *Sst* expression) that targeted different GABAergic, dopaminergic or GABAergic/dopaminergic neurons in the VTA and in the DRN by combining retrograde Rabies tracing with fluorescent in situ hybridisation (FISH).

3.5 PAPER I - A HYPOTHALAMUS-HABENULA CIRCUIT CONTROLS AVERSION

3.5.1 AIMS

The aim of this study was to examine the glutamatergic input to LHb from the LH/GPi border region and to determine their role in aversive behavior and decision-making. We have combined a large number of state of the art techniques to answer this question.

3.5.2 RESULTS AND CONCLUSIONS

In an effort to identify molecular characteristics of input neurons from LH and GPi to LHb, rabies tracing experiments were performed in *Vglut2-Cre:TVA* mice. A two-virus retrograde tracer was injected into the LHb of these mice and resulted in strong labelling in the LH and GPi areas. To identify the molecular composition of these projecting neurons, we performed snRNA-seq of *Vglut2*-positive nuclei from these regions. One important aspect of the analysis of these data was to identify GPi-specific cells that co-expressed *Vgat* (a marker for GABAergic neurons) and *Vglut2* (a marker for glutamatergic neurons) and to identify a unique molecular signature for these specific GPi-LHb neurons. We found that this population also expressed *Sst* (Somatostatin, an inhibitory neurohormone). This allowed us to distinguish between GPi and LH glutamatergic projection neurons, since cells with co-expression of *Vgat* and *Vglut2* and *Sst* were found in the GPi but not in the LH. We visualized these cells using smFISH methods and were able to confirm their specific localization to the GPi. Furthermore, rabies tracing experiments revealed diverging inputs onto the glutamatergic neurons in GPi versus LH. The GPi received inputs from limbic areas while the LH population received sensorimotor input. To identify the role of these distinct glutamatergic projection neurons in regulating the encoding of aversion signals, optogenetic manipulations were used to probe the function of the identified neuron populations. We found that activation of glutamatergic LHb-projecting neurons from LH, but not from GPi, induced a strong aversive behaviour in a place-preference paradigm. A probabilistic two-choice task revealed that activation of glutamatergic LHb-projecting neurons from LH, but not from GPi, could decrease the value of a performed action. Importantly, imaging in vivo of the neuronal activity of the LH-LHb neurons using microendoscope imaging of the genetically-encoded calcium sensor GCaMP6, revealed that the LH-LHb *Vglut2* neurons signaled aversive events and rapidly developed a predictive signal of the CS-US (conditioned stimulus – unconditioned stimulus) association during fear conditioning.

3.6 PAPER 4: MOLECULAR DIVERSITY OF HABENULA AND LATERAL HYPOTHALAMUS NEURONS PROJECTING TO SEROTONERGIC NEURONS IN THE DORSAL RAPHE NUCLEUS AND DOPAMINERGIC NEURONS IN THE VENTRAL TEGMENTAL AREA

3.6.1 AIMS

The goal of this study was to characterize the neurons from habenula and LH that project monosynaptically to DRN and VTA. A lot of tracing studies have linked these brain regions and show the magnitude of connectivity. Other studies have focused on transcriptomics of the HC and LH in general. We used state of the art techniques to combine these two approaches. We used two-virus retrograde tracing

to introduce a GFP tag into the nuclei of projecting neurons and used this tag to isolate these nuclei and sequence their individual transcriptome.

3.6.2 RESULTS AND CONCLUSIONS

We were able to show that HC and LH projection neurons to DRN or VTA show distinct molecular characteristics. The HC projection neurons could be traced back to their subnuclei of origin based on the expression of known markers for either MHb or LHb. LHb neurons target predominantly the DRN, with only 20% connecting to VTA. The MHb, however, does not project to DRN, a finding that is in accordance with results of earlier tracing studies (Pollak Dorocic et al., 2014). In addition to the clear separation between subnuclei, we did find a cluster of cells that express markers for both. It has been suggested that there is a “transition zone” between LHb and MHb, called HbX. Our data support that suggestion and indicate that cells from this zone project to DRN. While all projecting neurons from the HC are glutamatergic, projection neurons from the LH are partly GABAergic and partly glutamatergic. Both populations project to DRN and VTA. Indeed, there does not seem to be any clear clustering of the LH data based on projection target.

We observed an interesting divergence in the expression of 5HT-, DA- and cholinergic receptors in both HC and LH. While DA- receptor expression was sparse in both populations, the LH GABAergic projection neurons in LH are the only cells expressing the D2 receptor gene. The 5HT-receptor expression was more abundant. *Htr2c* is a known marker gene for the LHb and is almost completely absent in the MHb. However, the MHb expresses *Htr5b*, which is only sporadically found in the LHb and completely absent in the LH. It is important to note that this 5HT receptor is non-functional in humans, but not in mice. The LHb and MHb differ greatly also in expression of cholinergic receptor genes with the MHb expressing nAChRs (nicotinic acetylcholine receptors) and the LHb mAChRs (the muscarinic type). This is in line with the fact that the MHb is heavily implicated in the effects of nicotine, as described earlier. These results show a great diversity in the molecular composition of HC and LH neurons projecting to DRN and VTA, indicating an intricate functional network between these structures.

CHAPTER 4

4 SINGLE-CELL RNA-SEQUENCING

Single-cell sequencing (SCS) is a scientific tool that has emerged about a decade ago (Tang et al., 2009). It has greatly enhanced our understanding of the diversity of cell types, facilitates the understanding of tissue and cell diversity in health and disease, and enables the development of new treatments (Stark et al., 2019). Before SCS was widely accessible and affordable, bulk sequencing was the standard technique, which was much more limited. Currently existing SCS technologies can be divided into well-based and droplet-based isolation methods (Ziegenhain et al., 2017). Well-based methods rely on high-throughput fluorescence-activated cell sorting (FACS), low-throughput laser capture microdissection (LCM) or manual isolation. The latter two are labor-intensive with comparably little yield but allow for inclusion of spatial information, a feat that FACS does not provide. Droplet-based isolation relies on stochastic distribution of single cells in droplets. While LCM, for example, allows for very specialized selection of cells (Datta et al., 2015), it is not suitable to isolate adult neurons. Adult neurons can have hundreds of branches that three-dimensionally span the brain tissue in different directions. LCM damages large portion of neurons and while the loss of cell content might be acceptable in some cases, the technique itself can introduce damage to mRNA molecules (Humerick et al., 2013). Therefore, a large number of studies have used FACS for the isolation of single neurons. This method can purify a sample based on different metrics like emission profile, size or granularity (Cuevas-Diaz Duran et al., 2017). There are a number of different sequencing techniques available today and an in-depth review would be beyond the scope of this thesis. Because of the relevance in the studies included in my thesis, I will focus on the SmartSeq2 method. For further review on the other methods, see (Ziegenhain et al., 2017) and (Svensson et al., 2018).

4.1.1 SMARTSEQ2

This technique is an improved version of an earlier established sequencing protocol, SmartSeq (Zhu et al., 2001) and , was first introduced in 2013 (Picelli et al., 2013) and has gained popularity since then because of its sensitivity and accuracy. Similar to other techniques, the SmartSeq2 workflow starts with FACS-sorting of lysis of the single cells or nuclei into pre-made lysis buffer. The lysed suspensions are provided with oligo-dT primer that binds to the polyadenylated (polyA) tails of the mRNA. The mRNA is then reverse transcribed followed by a template switch step. This step switch allows for rapid amplification of the cDNA obtained from the original template. The cDNA library is then sequenced in a with regular high-throughput sequencer standardized apparatus (Illumina HighSeq

2000 in our studies). An overview of the workflow can be seen in figure 4 (from Picelli et al., 2014).

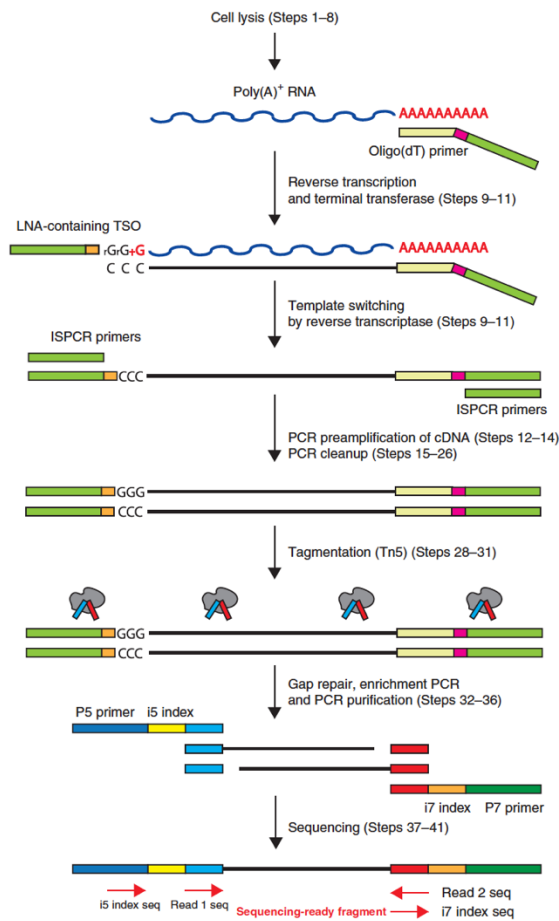


Fig.4: Flowchart for Smart-seq2 library preparation. Outline of the protocol and the corresponding procedure steps. The oligo-dT primer, TSO and ISPCR primer are described in the main text, whereas tagmentation uses primers that are included in the Nextera XT sample preparation and index kits. (Image and legend from Picelli et al., Nat Protoc 9, 171–181 (2014))

4.2 SINGLE NUCLEI VS. SINGLE-CELL RNA SEQUENCING

A method that has received attention in the last couple of years is single-nuclei RNA sequencing (Grindberg et al, 2013). As the name suggests, instead of isolation of single cells the nuclei of cells are isolated, sorted and sequenced. One major advantage of this technique is the improved dissection process. It is faster to isolate nuclei than cells from complex tissue, especially the brain. Importantly, some neuron types are sensitive to tissue dissection and consequently underrepresented in studies of single cell isolates (Tasic et al., 2018). Another caveat of single neuron isolation is that non-neuronal brain cells are included in the final cell suspension, which may not be of interest (Darmanis et al., 2015). A major problem of single nuclei isolation is the loss of cytoplasmic mRNA (Lake et al., 2017, Bakken et al. 2018). A recent study provides a comprehensive comparison between snRNA and scRNA sequencing techniques and concludes that the number of transcripts is larger from whole cell preparations compared to the nuclei sample (11.000 vs 7000 transcripts), with comparable power to identify cell types in both approaches (Bakken et al., 2018). This is in accordance with our observations. We compared single-nuclei RNAseq datas from Vgat+ neurons from the PFC with a public dataset from single Vgat+ neurons from the visual cortex (V1) and found that the library

sizes differed between 9000 for the whole-cell assay and 5500 for the nuclei. For the reasons mentioned, we chose to use single nuclei preparations for my PhD project to identify cell types. We labeled neuronal nuclei with a molecular construct that incorporates a fusion between GFP and histone 2B in a Cre-dependent manner. This way we isolated nuclei of a brain region of interest, sorted and sequenced only GFP+ nuclei (see Abe et al. 2011).

4.3 SINGLE NUCLEI RNA SEQUENCING ANALYSIS

As the sequencing techniques rapidly improved, so did the analysis tools for the resulting data in the last couple of years. One main issue that raised with single cell sequencing was the problem to normalize the data. Amplification of the mRNA during the preparation process introduces noise in the data. The introduction of RNA spike-ins during the preparation protocol allows for improved normalization. The ERCC spike-ins, developed by the External RNA Controls Consortium are polyadenylated transcripts with varying length (between 250 and 2000 nt) and are added in identical volume to each individual lysed cell solution (External RNA Controls Consortium, 2005; Pine et al., 2016). Sequencing the resulting ERCC spike-ins is then used to interpret technical variance that occurred during the remaining of the protocol. The versatile applications of single cell sequencing make a generalization of analysis methods impossible. However, this fact can be seen as a blessing rather than a problem because there are different methods of analysis for the various aims that a study may have.

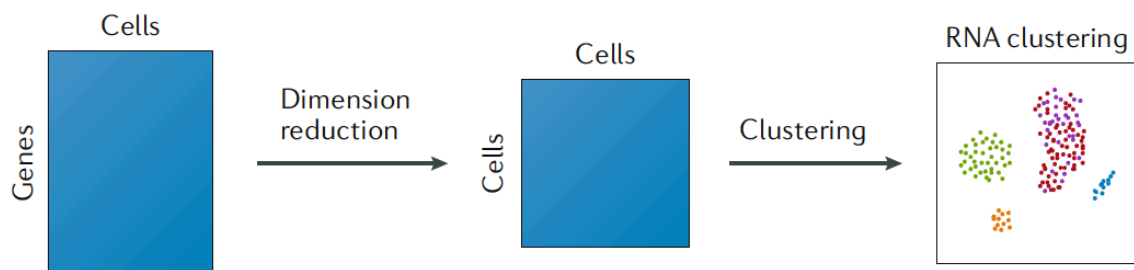


Fig. 5: Quick summary of the analysis process that starts with a gene-cell matrix. Dimensionality reduction leads to cell-cell correlations that can then be clustered. These clusters are defined by markers that can be identified using differential expression methods. (Image modified from Stuart and Satija, Nat Rev Genet. 2019)

The projects described in my thesis aimed at identifying subpopulations in known neural populations. We followed a path of analysis that included stringent quality control based on library size, number of genes expressed, size factors and ERCC ratios to eliminate sub-par data quality. In paper 4, we introduced a nuclear GFP tag using a Rabies viral tracing method. This virus, while genetically modified, still exhibits some cytotoxicity. Indeed, we saw cells with high levels of heat shock protein expression. Therefore, we added another QC step and eliminated these cells in this study.

After passing the QC, variable expressed genes were identified based on their dispersion in the sample population. Principal component analysis was used to identify genes with a significant expression variance. Based on these, another dimensionality reduction was performed using t-Stochastic Neighbor Embedding (tSNE, van der Maaten & Hinton, 2008), which formed the basis for density-based clustering of the data. Subsequently, differentially expressed (DE) genes for each cluster were identified. These DE genes marked putatively new populations within the neural populations. Currently, there is a large number of computational tools available for data analysis and the methods improve constantly. For the projects included in this thesis, we used a combination of freely available R tools, with Seurat used as the main package (Butler et al., 2018). The Seurat pipeline contains comprehensive and appropriate statistical analysis functionality and means to visualize the data.

4.4 SPATIAL TRANSCRIPTOMICS

In 2016, a method became available that solved several important problems in the sequencing methodology: It allowed for high-throughput RNA sequencing while preserving spatial information of the recorded transcriptomes. The technique allows for RNA capture in intact tissue on a prepared glass slide. The glass surface contains individual dots that are each spotted with millions of primers that include spatial barcodes and an RNA capture site. The primer design enables cDNA library preparation of captured mRNA, with the cDNA including the spatial information. Frozen tissue sections are placed on the dots and imaged, so that sequenced transcripts can be located on the imaged tissue slide (Ståhl et al., 2016).

4.5 PAPER 3 - MOLECULAR ATLAS OF THE ADULT MOUSE BRAIN

4.5.1 AIMS

The goal of this study was to create a gene expression map of the whole mouse brain. In order to do this, we utilized the technique developed by the Lundeberg and Frisén lab that is described above. In brief, fresh frozen 10 μ m brain tissue sections were transferred to a glass slide that contained an array of 1000 spatial dots. The dots contain millions of reverse transcription primers with unique positional barcodes that allow imaging of the tissue, capturing of mRNA with preserved spatial information and sequencing of mRNA. This design allowed us to identify in an unbiased way the transcriptome and spatial distribution in the adult mouse brain. Starting at the tip of the PFC and finishing at the cerebellum, we sequenced 75 coronal sections spanning the entirety of the mouse brain. In other words, we have produced a massive amount of data from virtually all regions in the mouse brain.

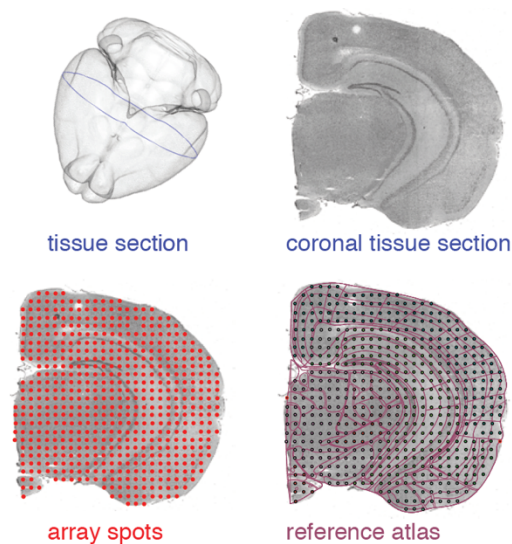


Fig. 6: Example of image processing pipeline (section at -2.88 mm posterior to bregma) showing the section outline in 3D (top left), the HE stained image (top right), the alignment of the HE image with the ST spots array (bottom left), and alignment with the mouse brain reference atlas (ABA, bottom right). Image from paper 3: Cortiz et al, Molecular Atlas of the Adult Mouse Brain, bioRxiv 784181; doi: <https://doi.org/10.1101/784181>)

4.5.2 RESULTS AND CONCLUSIONS

After strict quality control, our data set contained 15,326 genes and 34,053 spatial dots. We aligned the obtained images of each section to the Allen Brain reference atlas. This demonstrated that the molecular clustering in our data followed known neuroanatomical structures.

We created a whole-brain mouse atlas that combines molecular properties with spatial information. Two examples highlighted the usefulness of this atlas. First, molecular clusters in the isocortex were separated according to the barrel layer they were located in, with marker expression according to the published layer structure. Moreover, we identified gene expression patterns that organize the cortex in anterior-posterior and mediolateral directions.

The second important finding was the discovery of the brain palette; a subset of genes that is sufficient to classify the organizational complexity of the whole mouse brain. Availability of this information greatly reduced computing time and improved the molecular characterization. Consequently, it facilitates the identification of additional area specific palettes.

The atlas we created in this project is freely accessible to the public and has the potential to become a valuable reference for neuroscience research.

5 CONCLUSIONS

Neuroscience is arguably one of the most fascinating fields of research, has gripped me since high school and never let go. Thanks to constant technological advances, we have come a long way and it will be elating to see what is yet to come. Unraveling the neural basis of behavior is an ambitious goal and the more information we collect, the more complicated it gets. The work presented in this thesis can only be tiny fraction but hopefully an important piece of the puzzle.

The research into neural circuits that control behaviors is quite diverse and multifaceted. This is demonstrated in the studies discussed in this thesis. In paper 1, we combined tracing, electrophysiological, histological, molecular and behavioral experiments to show that the LH-LHb glutamatergic pathway is the one regulating aversive behaviors. In paper 2, we relied heavily on molecular, histological and statistical methods to reveal new subpopulations in the striatum, a structure that is critical not only for the regulation of movement, but also for reward, aversion and decision-making. The results of paper 3, where we used state of the art technology of spatial transcriptomics on a massive scale, have the potential to become an additional reference pool for neuroscientists. And finally, in paper 4 we used combined viral, tracing and sequencing techniques to molecularly define the neurons that target monoaminergic centers in the brain. All these studies add information about the brain and how behavior is regulated.

The aim of medical sciences, is to develop treatments for disease and disorder, and to find new solutions that promote wellbeing. Behavioral disorders are plenty and some are a growing problem in society because of the sheer mass of affected people. In neuroscience, we identify the sources of the disorders and aim to find the best way to help patients. The rapid improvement of single cell RNA sequencing techniques allowed researchers to identify neurons based on their molecular profile. This knowledge makes it possible to identify specific cells that contribute to malfunctioning and are linked to disease. Eventually, the acquired knowledge will aid in the discovery of targeted pharmaceutical countermeasures that not only alleviate the symptoms of a behavioral disorder, but also leave the rest of the system unaffected. Neuroscience will become more effective and new findings will help us understand the functions and malfunctions of the brain.

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