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ORGANIZATION OF BRAIN CIRCUITS THAT CONTROL MOTIVATED BEHAVIORS

Ourania Tzortzi

Karolinska Institutet

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Organization of brain circuits that control motivated behaviors
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ourania Tzortzi

Principal Supervisor:
Associate Professor, Konstantinos Meletis
Karolinska Institutet
Department of Neuroscience

Co-supervisors:
Professor, Gilberto Fisone
Karolinska Institutet
Department of Neuroscience

Professor, Johan Franck
Karolinska Institutet
Department of Clinical Neuroscience

Opponent:
Assistant Professor, Yeka Aponte
Johns Hopkins University
Department of Neuroscience

Examination Board:
Associate Professor, Karima Chergui
Karolinska Institutet
Department of Physiology and Pharmacology

Associate Professor, Andrea Carmine Belin
Karolinska Institutet
Department of Neuroscience

Associate Professor, Anna-Lena Ström
Stockholm University
Department of Biochemistry and Biophysics

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Μελετᾶν οὖν χρὴ τὰ ποιοῦντα τὴν εὐδαιμονίαν, εἴπερ παρούσης μὲν αὐτῆς πάντα ἔχομεν, ἀπούσης δὲ πάντα πράττομεν εἰς τὸ ταύτην ἔχειν.

Είναι λοιπόν χρήσιμο να μελετάμε αυτά που φέρνουν την ευδαιμονία, καθώς έχουμε τα πάντα όταν υπάρχει, όμως όταν αυτή λείπει κάνουμε τα πάντα για να την αποκτήσουμε. Επίκουρος

We must wonder ourselves on what brings eudemonia (happiness), for if we have it we have everything, but when it's absent we would do anything to have it back. Epicurus

To Paschalis

Organization of brain circuits that control motivated behaviors

Ουρανία Τζώρτζη

ABSTRACT

Eudemonia (Greek: εὐδαιμονία) is an anthropocentric term describing the absolute well-being in the Aristotelian ethics, along with the terms "arête" (virtue) and "phronesis" (wisdom from an ethical and practical point of view). Arête and virtue will guide ones decisions to promote future eudemonia and ultimately survival. Some of the classic parameters that shape decisions across species include the ability to experience reward, the good prognosis of reward and the avoidance of events that would harm ones physiology and psychology. Such behavioral complexity is orchestrated by a plethora of brain circuits.

A well-established candidate mediating reward-related behaviors is the neurotransmitter dopamine and the dopaminergic pathways. The activity of the dopaminergic pathways is influenced and modulated by cortical and subcortical areas involved in pain, mood regulation, arousal, stress and substance abuse (Hikosaka et al., 2008). These neuronal networks, affecting directly or indirectly the dopamine activity, are shaping motivated behaviors and decision-making.

The conceptual aim of my thesis is to understand molecular, anatomical and functional features of brain reward circuits. In this thesis I will be summarizing the background literature of my projects with special references to the brain areas including the basal ganglia structures, the habenula complex and the parabrachial nucleus, in the context of motivation, anhedonia and pain.

In **Chapter 1**, the brain-reward system will be discussed, with references to the old and new methodologies used to study its neurobiology. The diverse modulatory effects of dopamine and its implications in the prediction of reward are also described here.

Chapter 2 reviews the basal ganglia literature. The neurochemistry and neuroanatomy of the main elements of basal ganglia are described here, with the main focus being on the area of striatum. The physiology and pathophysiology of striatal circuits are central in the control of motor, cognitive and limbic functions. Relevant to this chapter are the **Papers I and II.**

The work in **Paper I**, describes the cell-type specific corticostriatal projections as well as the molecular discrimination of the cortical layers. It will be also examined here, the acute-cocaine-exposure effect in motor and reward function on a molecular and behavioral level. During this study, an open-access pipeline was developed in order to easily identify and map neuronal features (cell bodies, axons, dendrites) on a standardized brain atlas.

Paper II deals with the complexity of striatal anatomy and neurochemistry. This work describes how cell-type-dependent and cell-type-independent spaces uniquely constitute the striatum. There are special references to a newly identified striatal medium spiny projection neuron.

In Chapter 3, the epithalamic structure of lateral habenula is reviewed on an anatomical and functional level. The general focus of the chapter is on how the lateral habenula is mediating anti-reward signals through its connectivity patterns and its direct effects on neuromodulatory systems. **Paper III** is related to this chapter.

The **Paper III**, reveals how the lateral habenula-mediated aversive and fear behaviors are reflected on a network connectivity level. Central in this project are the adjacent populations of the basal ganglia-hypothalamus borders that are of distinct neurochemistry and connectivity and also convey differential anti-reward signals to the lateral habenula.

Chapter 4, reviews the parabrachial nucleus structure and function. It is discussed here, the importance of the parabrachial activity in conveying internal and external signals (pain, fear, visceral malaise) to forebrain in order to promote decision-making. The last **Paper IV** (manuscript) relates to chapter 4.

Paper IV, deals with the unique cytoarchitecture of the lateral parabrachial nucleus and its afferent and efferent connectivity. The main focal point in this work is the previously undescribed cholinergic subpopulation of the nucleus, which displays both classic and distinct neuroanatomical features when compared with the well-established markers of the area.

In summary, this thesis captures the diversity of neuronal substrates scattered across the brain and yet function in the direction of maximizing well-being. Common substrate of these structures is their direct or indirect connections with the dopaminergic centers and the extended brain reward circuit. Paradoxically, activation of most of the areas examined here is primarily signaling aversion, fear and pain rather that reward! The combinatorial view when studying advance behavioral aspects leads to a finer comprehension of the physiological function.

LIST OF SCIENTIFIC PAPERS

I. An interactive framework for whole-brain maps at cellular resolution
Fürth D., Vaissière T., **Tzortzi O.**, Xuan Y., Märtin A., Lazaridis I., Spigolon G., Fisone G., Tomer R., Deisseroth K., Carlén M., Miller C.A., Rumbaugh G., Meletis K.
Nature Neuroscience. 2018 Jan;21(1):139-149.

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.e5.

III. A hypothalamus-habenula circuit controls aversion
Lazaridis I., **Tzortzi O.,** Weglage M., Märtin A., Xuan Y., Parent M., Johansson Y., Fuzik J., Furth D., Fenno L.E., Ramakrishanan C., Silberberg G., Deisseroth K., Carlén M., Meletis K.
Molecular psychiatry. 2019 Sep;24(9):1351-1368.

IV. Characterization of a cholinergic-glutamatergic population in the lateral parabrachial nucleus
Tzortzi O., Mantzafou A., Meletis K.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AAV	Adeno associated virus
APN	Anterior pretectal nucleus
BST	Bed nucleus of stria terminalis
cc	Corpus callosum
Cck	Cholecystokinin
CEA	Central amygdalar nucleus
CGRP	Calcitonin G related protein
Chat	Choline acetyltransferase
CP or CPu	Caudoputamen
EPN	Entopeduncular nucleus
fr	Fasciculus retroflexus
GPb	Border region of the globus pallidus
GPe	Globus palidus external segment
GPi	Globus palidus internal segment
hc	Habenular commissure
IC	Inferior culiculus
IPN	Interpeduncular nucleus
LHA	Lateral hypothalamic area
LHb	Lateral habenula
LPO	Lateral preoptic area
MD	Mediodorsal nucleus of the thalamus
MHb	Medial habenula
MOR	μ Opioid receptor protein
MPO	Medial preoptic area
MSN	Medium spiny neuron
N3	Oculomotor nucleus
NAc	Nucleus accumbens
NDB	Diagonal band nucleus
NLL	Nucleus of the lateral lemniscus
NO	Nitric oxide synthetase
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
Oprm1	μ Opioid receptor gene
ORB	Orbital area
Oxtr	Oxytocin receptor
PACAP	Pituitary adenylate cyclase
PAG	Periaqueductal gray
PB	Parabrachial nucleus
PBG	Parabigeminal nucleus
PBld	Parabrachial nucleus, dorsal lateral subdivision
PBle	Parabrachial nucleus, external lateral subdivision
pc	Posterior commissure
Pdyn	Prodynorphin
PF	Parafascicular nucleus
PPN	Pedunculopontine nucleus
PRN	Pontine reticular nucleus
PT	Pretectum
Pul	Pulvinar
PVAB	Parvalbumin
RMTg	Rostromedial tegmental nucleus
RNA-FISH	RNA fluorescent in situ hybridization
SC	Superior culiculus
SI	Substantia innominata
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulate
snRNA seq	Single-neuron nuclei RNA sequencing
SSp	Primary somatosensory area
Sst	Somatostatin
STN	Subthalamic nucleus
Tac1	Tachykinin precursor 1
TAN	Tonically active neurons
Th	Thalamus
VAL	Ventral anterior-lateral thalamus
Vgat	Vescicular GABA transporter
Vglut2	Vescicular glutamate transporter type 2
VM	Ventromedial nucleus of the thalamus
VMH	Ventromedial hypothalamic nucleus
VPM	Ventral posteromedial nucleus of the thalamus
VTA	Ventral tegmental Area
Zi	Zona inserta

1

THE BRAIN REWARD SYSTEM

1.1 INTRODUCTION

The reward system refers to a set of interconnected brain areas implicated in the performance of reward seeking and avoidance of punishment through incentive learning and motoric activity (Berridge et al., 2016; Hamid et al., 2016; Berke, 2018). Areas involved in this system include forebrain limbic structures and their links to the midbrain (i.e. dopaminergic and serotonergic) centers (Russo and Nestler, 2013). As a highly evolved neurobiological system it contains mechanisms for decision-making based on the identification, processing and evaluation of environmental stimuli to promote future eudemonia (Shizgal et al., 2001; Berthoud and Müinzberg, 2011). The ability to experience the diversity of rewards is essential to a healthy psychology of individuals. In reverse, affective disorders are commonly characterized by anhedonia or excessive dysphoria (depression, anxiety, fear, pain etc). Therefore, affective neuroscience aims to understand the neurobiology of reward system in order to eventually identify fine treatments for affective disorders (Haber and Knutson, 2010; Kringelbach and Berridge, 2010; Panksepp, 2011; Damasio and Carvalho, 2013; Heller et al., 2013; Anderson and Adolphs, 2014).

The first pioneer method for studying the reward system was the electrical brain stimulation (or intracranial self-stimulation, ICSS) (Olds and Milner, 1954). ICSS in brain areas located around the medial forebrain bundle (MFB) was the classical generator of reinforcing behaviors (Gallistel et al., 1981; Phillips and Fibiger, 1989; Wise and Rompré, 1989). As the field evolved, a plethora of anatomical regions beyond the mesolimbic dopamine system were promoting similar operant behaviors (Gallistel et al. 1981; Phillips and Fibiger, 1989; Ikemoto, 2010; Vlachou and Markou, 2011). Despite being a powerful tool, ICSS lacked specificity on a molecular and circuit level (Ikemoto, 2010; Vlachou and Markou, 2011). These limitations were overcome when the era of optogenetics in combination with advances in mouse genetics arrived. The technology of optogenetics is based on the expression of light-activated microbial opsins in neurons using genetic strategies (Zhang et al., 2007). The optogenetic approach allows for the bidirectional modulation of neuronal activity in defined neuronal populations with temporal precision during active animal behavior. The new technology allows for anatomical and functional dissection of the brain reward system and ultimately the motivational function.

1.2 A NEUROSCIENCE OF REWARD AND THE DOPAMINE HYPOTHESIS

A great variety of rewards can trigger overlapping neuronal networks. An essential and common element of the limbic system is dopamine and its dopaminergic projections to striatum (MFB). Dopamine has a multidimensional role in several neurobiological systems ranging from control of movement, motivation and reward-based learning, substance abuse, attention and mood regulation. DA imbalance is a common feature in the pathogenesis of diseases like schizophrenia, Parkinson's disease, attention deficit hyperactivity disorder (ADHD) and drug-addiction. The main dopaminergic pathways include a. the dopaminergic pathway from the ventral tegmental area (VTA), which targets the limbic ventral striatum such (NAc: nucleus accumbens) and b. the nigrostriatal pathway from substantia nigra pars compacta (SNc), which targets the dorsomedial striatum (DMS), dorsolateral striatum (DLS) and the tail of the striatum (TS) (Figure 1A).

The physiology and pathophysiology of the VTA-NAc and SNc-Striatum DA signaling is crucial for motivated behaviors (Phillips et al., 2003; Day et al., 2007; Stuber et al., 2008; Oleson et al., 2012), salience, stimulus-reward association learning (Koob and Volkow 2010; Gardner, 2011), pain (Xie et al., 2014), processing of nociceptive peripheral stimuli (Elman et al., 2013) and reward prediction error (RPE: the difference between the actual upcoming reward and the experienced reward) (Knutson and Cooper, 2005). Inhibition of DA neurons underlies behaviors associated with negative RPEs (Schultz, 2007) or learning in response to aversive stimuli (Ungless et al., 2004). Rewarding stimuli and their predictive cues cause phasic excitation of DA neurons, while aversive stimuli and reward omission lead to the phasic inhibition of DA neurons. (Schultz et al., 1997; Ungless et al., 2004; Pan et al., 2005; Tobler et al., 2005; Matsumoto and Hikosaka, 2007; Cohen et al., 2012). In response to salient stimuli the firing rate of VTA DA neurons increases and phasic dopamine is released in the ventral striatum (NAc) (Figure 1B).

Optogenetic efforts to excite DA neurons of VTA or SNc, have undoubtedly confirmed that such activation is reinforcing (Tsai et al., 2009; Witten et al., 2011; Kim et al., 2012; Ilango et al, 2014a; Ilango et al, 2014b; Pascoli et al., 2015; Stauffer et al., 2016), and also supports Pavlovian (Steinberg et al., 2013; Sharpe et al., 2017; Saunders et al., 2018), contextual (Tsai et al., 2009; Ilango et al, 2014b) and operant learning (Adamantidis et al., 2011; Witten et al., 2011; Hamid et al., 2016).

In reverse, optogenetic inhibition of DA neurons represents negative RPE, which eventually leads to the extinction of an established Pavlovian conditioning (Chang et al., 2016), so that an animal will gradually avoid a previously preferred action (Hamid et al., 2016; Parker et al., 2016) and eventually conditioned place avoidance will be induced (Ilango et al, 2014b).

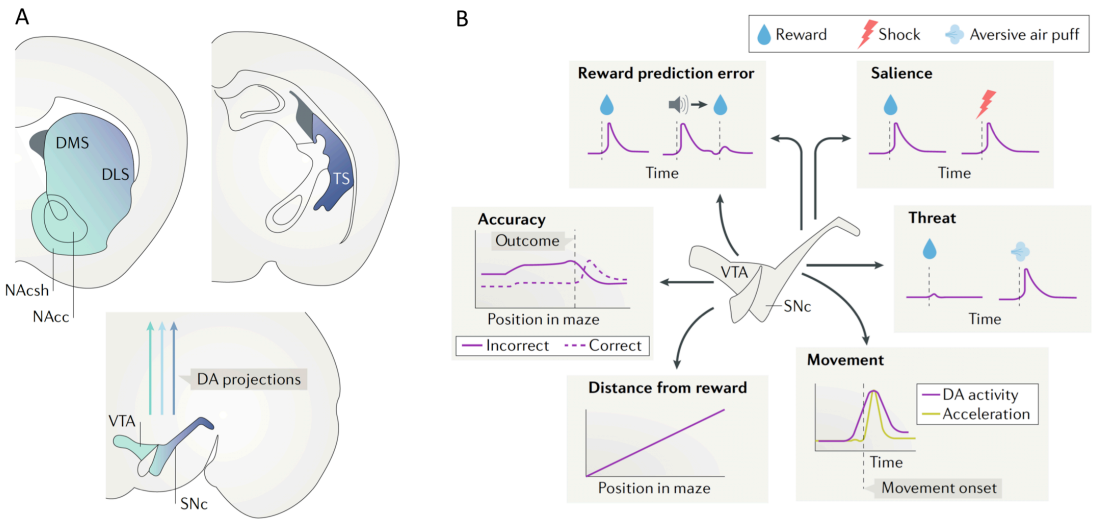

Figure 1. Diversity of midbrain dopaminergic projections to striatum. A Heterogeneous projection patterns of the midbrain dopaminergic nuclei (VTA; SNc). Color shading indicates the different striatal targets of VTA and SNc. Light blue: VTA to ventral striatum (NAc) dopaminergic pathway; blue: ventro-medial SNc to dorsal striatum dopaminergic pathway; dark blue: dorso-lateral SNc to tail of striatum (TS) dopaminergic pathway. **B** Examples of dopaminergic activity in VTA and SNc. Both VTA and SNc DA neurons signal Reward Prediction Errors by increasing their activity in response to reward (left) or in response to cues predicting the reward (right). Some medial VTA DA neurons are activated in response to the accuracy of the action. Other VTA DA neurons signal the proximity to the area where the reward is present. Changes in DA neuronal activity in the lateral VTA and the SNc are associated with movement. DA neurons of the lateral SNc are not signaling in response to reward but rather to salient and threat-related stimuli. Image from Cox, J., & Witten, I. B. 2019, Nature Reviews Neuroscience 20, 482–494.

1.3 THE OPIOID SYSTEM IN REWARD AND ADDICTION

Through its interaction with dopaminergic pathways, the opioid system is involved in the regulation of mood and addictive behaviors (Volkow, 2010; Charbogne et al., 2014). The opioid system is one of the main targets of several substances of abuse (i.e. heroin, cocaine, and alcohol) (Zubieta et al., 1996; Heilig et al., 2011). Opioid receptors and peptides are expressed primarily in cortex, limbic system and brainstem (Lutz et al., 2013). The highly addictive drugs of heroin and fentanyl are ligands of the μ opioid receptor (MOR), which is encoded by the Oprm1 gene (Fields, 2004). MOR is involved in regulating the rewarding properties of these drugs (Matthes, 1996), as well as mediating the antidepressant effects of tianeptine (Samuels et al., 2017) and buprenorphine (Robinson et al., 2017).

2

THE BASAL GANGLIA CIRCUITS

2.1 INTRODUCTION

The basal ganglia are a collection of strongly interconnected and evolutionarily conserved subcortical nuclei. The physiology and pathophysiology of basal ganglia are linked to the regulation of motor, cognitive and limbic functions. The main elements of the basal ganglia include the striatum, the globus pallidus internal and external segments, the substantia nigra and the subthalamic nucleus. The aforementioned forebrain structures form networks of open and closed loops circuitry with other brain areas involving among others the cerebral cortex, thalamus and brainstem (Nelson et al., 2014). The basal ganglia receive input from cortical areas and project back to other brain systems mediating the generation of behaviors. The main input to the basal ganglia system comes from layer 5 pyramidal neurons of the neocortex (glutamatergic input). The output structures of the basal ganglia are the substantia nigra pars reticulata (SNr) and the internal segment of the globus pallidus (GPi). The GABAergic output projections from GPi and SNr targets mainly thalamus, which projects to the cortex to promote planning and execution of motor behaviors.

2.2 THE STRIATUM

The process of decision-making requires a series or motoric actions based on sensory inputs and the internal reward evaluation. In this process the striatal circuits are essential in integrating sensory information and estimating the value of reward based on former experience, to ultimately promote learning and decision-making.

The striatum is classically characterized as the primary input nucleus of the basal ganglia. It receives direct cortical and thalamic input in order to shape motor learning based on current and past experiences (Graybiel, 2008), (Figure 2).

2.2.1 Striatal medium spiny neurons

The striatal medium spiny neurons (MSNs) neurons (GABAergic) can be differentially subdivided based on their molecular profiles, their topographic orientation, their functional interpretation and their projection patterns into five conventional categories:

1. The GPi and GPe projecting MSNs that give rise to the two opposing pathways of striatum: the direct and indirect striatal pathways respectively. The direct pathway promotes and the indirect pathway prevents movement (Albin et al., 1989). MSNs that belong to the direct pathway (dMSNs) are directly projecting and suppressing the neurons of the basal ganglia output structures of GPi and SNr (Figure 2A). The dMSNs are preferentially expressing the D1 receptor of dopamine along with substance P (SP) (Crittenden, 2011). The MSNs of the indirect pathway (iMSNs) project to GPe and indirectly enhance the basal ganglia output. The iMSNs express the D2 receptor of dopamine and proenkephalin (PENK) (Crittenden, 2011; Lee, 2016).

2. MSNs of the dorsal and the ventral striatum. Dorsal striatum is classically involved in motor control and receives its dopaminergic input from the SNc. Ventral striatum (VS; major component of VS is the nucleus accumbens; NAc) is frequently defined as the limbic part of striatum. The VS is mostly linked to reward-related behaviors and receives dopaminergic input from the VTA (Ikemoto, 2010). NAc has a central role in Pavlovian learning, involved in the formation of stimulus-outcome association and thus promoting motivational behaviors (Roitman et al., 2005; Balleine et al., 2009; Liljeholm et al., 2012). Due to its substantial communication with the midbrain dopaminergic centers (Groenewegen et al., 1999; Watabe-Uchida et al., 2012), NAc is a great modulator of DA release in striatum (Mannella et al., 2013).

3. MSNs of the dorsomedial (DMS; in rodents DMS is homologous to the putamen of the primates), dorsolateral (DLS;in rodents DLS is homologous to the caudate of the primates) striatum, which are often referred to as the associative and sensorimotor striatum respectively (Figure 2B). Lesion and optogenetic studies have indicated that the DMS striatum is necessary for goal-directed motor learning while the DLS striatum is more important in the development of habits (Thorn, 2010; Smith and Graybiel, 2013; Atallah, 2017). During learning both areas are active, but when over training a previously goal-directed action becomes more habitual, distinct activity patterns develop among DMS and DLS (Thorn et al., 2010; Yin et al., 2009).

4. The MSNs that give rise to patch (or strisome) and matrix compartments of striatum. Patches are 3 dimensional labyrinths embedded inside the matrix. Classically, immunolabeling for MOR (μ opioid receptor) defines the patches where cells positive for MOR also express the D1 receptor of along with other classical markers for D1 expressing neurons. However, matrix contains both D1 and D2 MSNs. Both compartments but mostly matrix receive dopaminergic innervation from SNc, while only patch MSNs project directly to SNc (Figure 2A). Moreover, patches receive inputs from limbic areas (Gerfen, 1984; Ragsdale and Graybiel, 1988; Eblen and Graybiel, 1995), in support to the hypothesis that patches convey motivational or emotional signals (Figure 2B). In this direction, rodents can easily learn to self-stimulate the

striosomes; however, this cannot be achieved when the stimulation takes place in matrix regions (White and Hiroi, 1998).

And 5. MSNs in the tail of striatum (TS). Even though this striatal subdivision is less studied, more and more evidence support the idea that TS is receiving sensory information (Znamenskiy et al., 2013; Sippy et al., 2015; Xiong et al., 2015; Chen et al., 2019).

2.2.2 Striatal cholinergic interneurons

The striatal cholinergic interneurons (or aspiny striatal neurons) express the neurontransmitter acetylcholine and are anatomically identified due to their large somata and their rich dendritic fields (DiFiglia et al., 1976; Chang et al., 1982; Bolam et al., 1984; Wilson et al., 1990; Kawaguchi, 1992). This striatal subtype is known to be tonically active (TAN neurons) (Kimura et al., 1984) and despite the fact that they constitute only 1-2% of the striatal neurons, they are of high importance (Kemp & Powell 1971). The cholinergic interneurons receive glutamatergic thalamic and cortical input (Lapper & Bolam 1992; Thomas et al. 2000) but also GABAergic projections from MSNs (Bolam et al. 1986).

2.2.3 Striatal GABAergic interneurons

The GABAergic interneurons of the striatum express GABA as a neurotransmitter and they have a medium-sized morphology (Bolam et al., 1983; Smith et al., 1987). They can be subdivided based on their molecular expression of neuropeptides (i.e. neuropeptide Y; NPY, somatostatin; Sst) and calcium-binding proteins (i.e. calretinin, parvalbumin; PVALB).

The largest subpopulation of the GABAergic striatal interneurons and is the fast-spiking PVALB-expressing interneuron (Cowan et al., 1990; Kubota and Kawaguchi, 1993). These neurons receive thalamic, pallidal and cortical input and they project to the striatal MSNs. PVALB interneurons are scattered throughout striatum but they follow a dorso-ventral gradient.

Other subtypes of GABAergic interneurons include those expressing Sst, nitric oxide synthetase (NO) and NPY (Smith and Parent, 1986; Dawson et al., 1991). The Sst-expressing interneurons display a ventral to dorsal gradient and most of them co-express NO and NPY (Tepper et al., 2010).

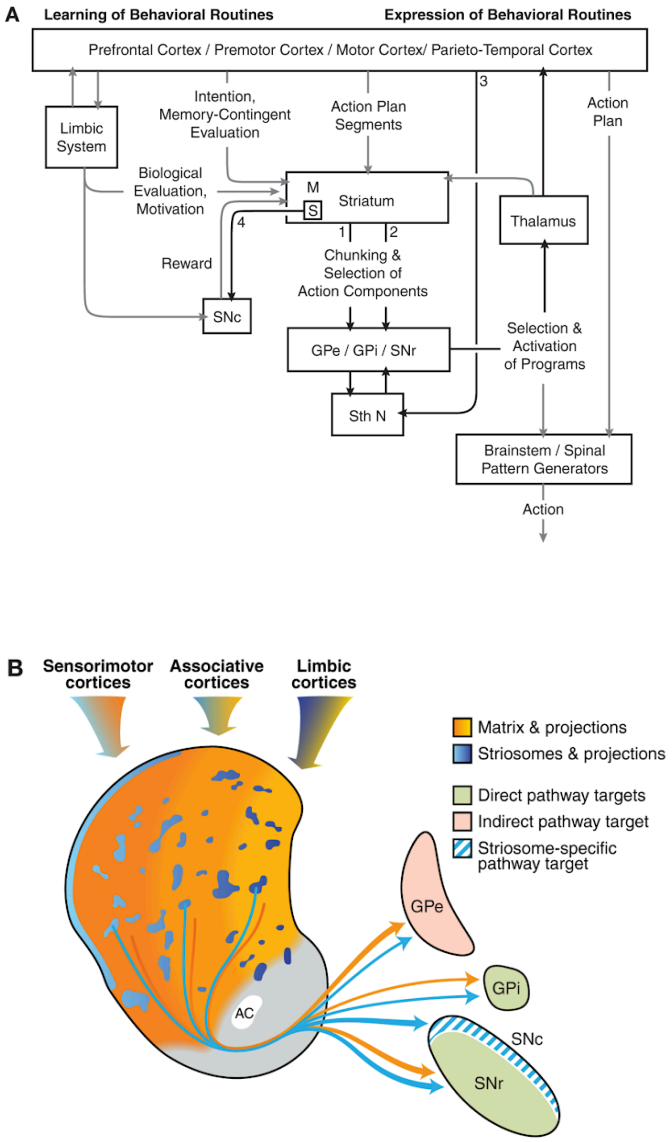

Figure 2. Neuroanatomy of the Cortico-Basal Ganglia-Thalamo-Cortical loops. A. Schematic of the main basal ganglia pathways. The behavioral implication of each pathway is indicated. The striosome (patch) and matrix striatal compartments are central components of the schematic. The four basal ganglia pathways emphasized here are: 1. the direct pathway (striatal D1-expressing MSNs directly projecting to GPi/SNr); 2. the indirect pathway (striatal D2-expressing MSNs indirectly projecting to GPi/SNr through the GPe); 3. the hyperdirect pathway (direct cortical input to STN); 4. the striosomal pathway (striosomal MSNs directly projecting to DA SNc neurons). **B.** Model of the main outputs of dorsal striatum to basal ganglia (projection pathways: direct in pink, indirect in green, striosomal in stripped blue). The striosome and matrix compartments of the DS are indicated in blue and orange respectively. The color-shading from the lateral (left) to the medial (right) DS refers to the sensorimotor, associate and limbic striatal areas respectively. The thickness of the arrows exiting the DS indicates the abundance of outputs to basal ganglia and the color of the lines represents striosomal (blue) and matrix (orange) striatal projections. The color-shading and shading of arrows entering striatum indicates the differential cortical input provided to the striosomal and matrix DS compartments: while sensorimotor and associative cortices project mostly to matrix; the striosomes receive inputs from limbic cortices. Image from: Crittenden J. R. 2011, Front. Neuroanat. *5*, 59.

2.3 THE GLOBUS PALLIDUS

The globus pallidus (GP) is located above and adjacent to the anterior commissure pathway and it is a structure involved in sensorimotor and associative processes (Kita, 2010). The GPe and GPi comprise the GP. The GPe lies medial to the striatum in rodents as well as in primates. The internal capsule circles the GPi and in rodents it is called the entopeduncular nucleus. Both GPe and GPi receive glutamatergic inputs from the STN, which receives excitatory input from motor cortices.

2.3.1 The GPe

The GPe is primarily receives GABAergic input from the striatum and glutamatergic input from the STN (the main input areas of basal ganglia). GPe sends GABAergic projections to other basal ganglia nuclei: GPi, STN, SNr and striatum (Kita, 2010). The prototypic and arkypallidal neuronal subtypes comprise the GPe population. These are two GABAergic GPe subpopulations characterized by distinct neurochemistry, connectivity and firing patterns (Mallet et al., 2012). GPe prototypic cell type innervates the STN, the basal ganglia output nuclei (GPi and SNr) and occasionally the striatum. The arkypallidal neurons project only to striatum (Mallet et al., 2012). The two subpopulations can be neurochemically identified: arkypallidal and prototypic neurons express the transcription factors FoxP2 and Nkx2.1 respectively. The Nkx2.1 prototypic neurons are further subdivided into those contain the calcium-binding protein parvalbumin (Nkx2.1/PV+) and those which do not (Nkx2.1/PV-) (Dodson et.al., 2015). When an animal is at rest state, the prototypic and arkypallidal neurons exhibit high and low firing rates respectively (Dodson et.al, 2015).

2.3.2 The GPi

The GPi lies posterior to the GPe (immediately posterior to the termination field of the indirect striatal pathway axons) and anterior to the STN in rodents. The GPi neuronal population contains both GABAergic and glutamatergic neurons capable of co-releasing GABA and glutamate (Shabel et al., 2014). The GABAergic population is spread throughout the anteroposterior axis of GPi. There is a Sst population situated in the more anterior GPi (Vincent and Brown, 1986) that also expresses and coreleases GABA and the glutamate transporter type 2 (Vglut2) (Lazaridis et al., 2018). The GPi along with the SNr are the output basal ganglia structures and receive inputs from the striatum, GPe, and brainstem areas, including the pedunculopontine nucleus (PPN). The GPi and SNr project back to STN and brainstem.

2.4 PAPER I

AN INTERACTIVE FRAMEWORK FOR WHOLE-BRAIN MAPS AT CELLULAR RESOLUTION

2.4.1 Aims and Purpose

The overall aim of this project was the development of an open source interactive software that enables the rapid detection, visualization, mapping and sharing of whole-brain data at a single cell level (http://wholebrainsoftware.org/), (http://openbrainmap.org). While the literature regarding basal ganglia connectivity, molecular diversity and behavioral implications is enormous, there is a growing need for whole-brain data handling software. During this project we aimed to discriminate the cortical layers through the differential expression of markers. Furthermore, we were interested in the whole-brain inputs to the main striatal populations focusing primarily on the corticostriatal connections. Then we performed a functional assay where the motoric effect of cocaine was translated into a whole-brain map of immediate early gene activity (c-Fos). Through this experiment we focus on the deferential activation of c-Fos in the dorsoventral axis of striatum but also across other limbic areas.

2.4.2 Methodological approaches

The whole-brain software allows for the handling of data generated by a variety of methodological approaches (Fürth et al., 2018). As a proof of concept, methods that were used in this paper involved gene-specific trans-synaptic viral tracing that allows for dissecting brain connectivity on a cellular and spatial resolution and also immunochistochemical and single-molecule fluorescent in situ hybridization (sm-FISH) approaches for characterizing the differential molecular profiles of single cell types as well as defining their quantities, distribution and localization on biological tissue.

More specifically, immunohistochemistry for the markers NPY and PVALB and was applied on an Lhx6:EGFP transgenic mouse line, and their single and combinatory expression across the cortical layers was examined. Then monosynaptic retrograde Rabies tracing was used to identify the corticostriatal projections inputs to the D1/D2 expressing MSNs as well as the cholinergic striatal population (D1-Cre, D2-Cre and Chat-Cre mouse lines were used). For the functional assay, two groups of wild-type mice were intraperitoneally administered either cocaine (20mg/kg) or saline. Both groups were assessed in the classic open field test and then sacrificed in order for the whole-brain c-Fos activation to be measured immunohistochemically.

2.4.3 Results of Paper I

The whole-brain software was first used to map the spatial distribution of an interneurons' set (Lhx6, NPY, PVALB) across the cortical layers and other anatomical regions. The datasets generated from the software, contained information regarding the singular and combinatorial distribution of the markers. The genetic targeting of Lhx6 (Lhx6::EGFP transgenic mouse line) in combination with immmunolabeling for the markers NPY and PVALB generated datasets with the distribution profiles of each marker and/or combinations of markers' co-expression with Lhx6. We confirmed that the markers NPY and PVALB do not overlap in the cortical Lhx6::EGFP population, while 73% of the PVALB+ neurons co-expressed Lhx6::EGFP and 37% of the NPY+ cells overlap with Lhx6::EGFP. Contrary to the cortex, the 84% of the striatal NPY+ population co-expresses Lhx6::EGFP. Furthermore, we investigated the laminar distribution of these markers. Five clusters were examined: 1. Lhx6; 2. NPY; 3. PVALB; 4. Lhx6/NPY; 5. Lhx6/PVALB. The cortical NPY+ and PVALB+ were mainly found in the primary somatosensory cortex (SSp) layer II/III, while the Lhx6+/NPY+ and Lhx6+ neurons were mostly on layer V.

In addition, we aimed to define the differential cortical input to the main striatal cell types: the D1 and D2 expressing MSNs as well as the cholinergic (Chat) neurons. Therefore, monosynaptic retrograde rabies tracing was applied (Wickersham et al., 2007), leading to the generation of cell-type specific mapping of the corticostriatal connectivity. We investigated the laminar distribution of the D1, D2 and Chat presynaptic cortical inputs. Projections to the D1+ and D2+ MSNs arose primarily from the SSp layers V and ll/lll. Inputs to striatal Chat+ neuros arose from the SSp layers V and VI. We also compared the cortical versus the subcortical inputs to these three striatal populations. We found that the D2+ MSNs received more cortical than subcortical input, while the D1+ MSNs and Chat+ neurons received almost equal amounts of cortical and subcortical efferents. These results revealed the distinct cortical and subcortical connectivity patterns with the distinct neuronal subtypes of striatum.

Last, we used our software to associate the cocaine-induced changes in mouse locomotion with immediate early gene activity on a whole-brain scale. Thus, we were able to correlate neuronal activity (measured by c-Fos expression) with cocaine-mediated motor behavior. Here, mice were administered a high dosage of cocaine acutely and thereafter the effect on locomotion was tested. Finally, the whole-brain c-Fos expression was examined with the main focus being on the corticostriatal circuits (activity mapped on the orbital area; ORB). As predicted, the cocaine-treated group displayed a significant increase in locomotion compared to the control group. The whole-brain c-Fos expression was also increased in the cocaine-treated group. Interestingly, we found that the cocaine did not alter the total numbers of c-Fos expressing cells in ORB but it rather induced an increase in their intensity levels, which was probably due to the increased locomotion. Areas characterized by increased c-Fos expression included the CPu and NAc. This activation was most probably due to the cocaine effects on the nigrostriatal and mesolimbic dopaminergic pathways respectively.

2.5 PAPER II

A SPATIOMOLECULAR MAP OF THE MOUSE STRIATUM

2.5.1 Aims and Purpose

The striatum is a basal ganglia structure implicated in motor function and reward processes. Several striatal-mediated behavioral aspects are attributed to specific subregions of the structure or to molecular distinct subpopulations. For example, the mesolimbic pathway mediates reward processes and involves the ventral striatum (NAc) and the nigrostriatal pathway mediates motor function through the dorsal striatum (CPu). During the classical learning processes a goal-directed action becomes more habitual and the neuronal activity underlying this process follows a mediolateral activation gradient in the dorsal striatum. Moreover, upon activation the D1 MSNs promote movement while the D2 MSNs prevent it.

Despite the tremendous amount of literature, the striatal spatial and molecular dissection beyond the classical knowledge is a necessity. In this direction the aim of this project is to describe the heterogeneous spatial and molecular resolution of the MSNs, in the context of striatal patch, exopatch and matrix compartments.

2.5.2 Methodological approaches

In summary, methods used in this study included single-neuron nuclei RNA sequencing (snRNA-seq), retrograde tracing with retrobeads, immunohistochemistry, single-molecule fluorescent in situ hybridization (sm-FISH), tissue clearing in combination with mouse genetics. Mouse lines used here include the Vgat-Cre and Oprm1-Cre to capture the whole GABAergic population of the dorsal striatum and the Oprm1-expressing striatal population of the patch/exopatch compartments respectively. Both lines were crossbred with reporter lines allowing for the anatomical identification of the aforementioned populations and enable nuclear isolation necessary for the snRNA-seq. Data generated by the snRNA-seq, were confirmed histologically with sm-FISH and immunohistochemistry. We were then able to capture the distinct expression patterns of snRNA-seq-identified unique striatal markers.

2.5.3 Results of Paper II

For this project, we used a novel knock-in transgenic mouse line that expresses the cre recombinase in the μ Oprm1-expressing neurons, so that we can specifically target the Oprm1+, patch and exopatch striatal populations. By combining the genetic targeting of Oprm1 (Oprm1:tdTomato transgenic mouse) with tissue clearing we were able to visualize the 3D morphology of striatal patch as well as exopatch population. We used an unbiased model where the topography of a single tdTomato+ neuron was determined by its proximity to the five closest tdTomato+ cells. The results indicated that the vast majority of tdTomato+ neurons (88,5%) belonged to the patch compartment while the rest constitute the exopatch population. We aimed to determine the specificity of the Oprm1:tdTomato mouse, by applying sm-FISH for the marker Oprm1. It was revealed that both patch and exopatch tdTomato+ neurons displayed Oprm1 expression. In the same direction, we further confirmed that Oprm1+/tdTomato+ neurons from both patch and exopatch compartment co-expressed markers for the D1 MSNs (Pdyn and Tac-1).

The next part of this study, aimed to map the RNA profiles of MSNs. Therefore, snRNA-seq was applied on dissected dorsal striatum tissue of Vgat-Cre:H2bGFP and Oprm1-Cre:H2bGFP mice. From the clusters generated, we could visualize that all nuclei expresses general MSN markers (for example DARPP-32) as well as cell-specific markers for the D1 and D2 MSNs (nuclei from the Oprm1 dataset: 87% expressed D1 and 9% expressed D2; nuclei from the Vgat dataset: 57% expressed D1 and 37% expressed D2). Additionally, small clusters displayed interneuronal identity (Sst/NPY; TH; PVALB).

For the molecular dissection of the patch and exopatch populations the candidate patch versus matrix clusters were examined. Patch clusters preferentially expressed the marker Sema5b and exopatch clusters displayed expression of the Id4 marker. These findings were confirmed histologically with sm-FISH on Oprm1:tdTomato mice. The majority of tdTomato+ patch neurons expressed Sema5b (negative Id4 expression), while the tdTomato+ exopatch population along with the rest of matrix cells expressed the marker Id4 (negative Sema5b expression), (Figure 3A). Thereby, the Oprm1+ striatal population can be further subdivided into the Oprm1+/Sema5b+ patch and the Oprm1+/Id4+ exopatch subpopulations. The rest of matrix cells also exhibited Id4 expression. The markers Sema5b and Id4 can be used to easily visualize the striatal patch and matrix compartments respectively, while the exopatch can be identified by the dual expression of the markers Oprm1+/Id4+.

Alongside with the large D1+ and D2+ clusters revealed by the snRNA-seq data, there was also a separate small D1+/DARPP-32+ cluster (molecularly distinct from the D2+ cluster) expressing the marker Col11a1. Sm-FISH on Oprm1:tdTomato mice revealed that the Col11a1+ nuclei have overlapping expression of Sema5b but not Oprm1 (Figure 3B). Additionally, Col11a1+ cells have higher Sema5b expression than the Oprm1+ patch cells. These neurons constitute probably a unique MSN subpopulation characterized by the distinct molecular profile (i.e. here Col11a1+ / Sema5b+/ Oprm1-) and distinct striatonigral Oprm1- projections.

For the last part of the project, we aimed to investigate how expression gradients observed in our datasets reflect on the striatal anatomical space. Three markers were identified from our datasets (Crym, Gpr155, Dlk1) and their spatial distribution was examined with sm-FISH in two anteroposterior coordinates of the Oprm1:tdTomato mouse. All markers displayed a cell-type-independent expression (their expression was present in both D1+ and D2+ clusters in the sn-RNA-seq datasets) and they seem to represent distinct striatal subspaces (Figure 3C). The histological results on the Oprm1:tdTomato mouse, revealed that the majority of tdTomato+ neurons of the medial striatum co-expresses Crym. On the contrary, is situ expression of Gpr155 was present in both tdTomato+ and tdTomato- neurons of the central and lateral striatum. Gpr155 expression pattern was distinct and non-overlapping with the Crym space in medial striatum. Last, a separate medioventral part of striatum was characterized by Dlk1+ expression. Similarly to Crym and Gpr155, the Dlk+ area was spatially separated from the other two. These results pinpoint that there is a striatal code beyond the classical cell-type defined one. This code is rather represented by unique spatiomolecular subdivisions of striatum of unexplored neurobiological implications.

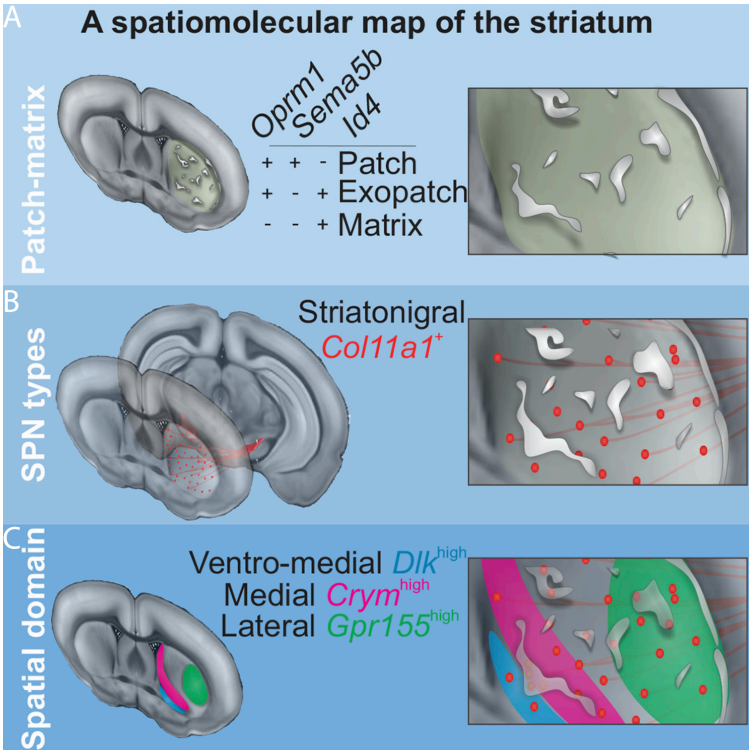

Figure 3. **The spatial and molecular subdivisions of the striatum. A** The differential expression patterns of the patch (Oprm1+/Sema5b), exopatch (Oprm1+/Id4+) and matrix (Oprm1-/Id4+) compartments of the striatum **B** The combinatorial expression of Col11a1+ / Sema5b+/ Oprm1- defines a separate subpopulation of striatal MSNs, which uniquely contributes to the striatonigral pathway. **C** The cell-type-independent spatial subdivisions of striatum. The expression patterns of the markers Dlk, Crym and Gpr155 define unique spaces of the ventro-medial, medial and lateral parts of striatum. This expression is continuous in the anteroposterior axis. Image from: Märtin, A. 2019, Cell Reports, 29(13), 4320–4333.e5.

3

THE LATERAL HABENULA

3.1 INTRODUCTION

The dosral dienchephalic conduction system (DDC) consists of the habenular complex, stria medullaris and the fasciculus retroflexus and is thought to interconnect the limbic and striatal forebrain with mid- and hindbrain areas. In this system the point of convergence is the habenular nucleus (Sutherland, 1982).

The habenular complex is situated in the epithalamus that is comprised of the medial (MHb) and lateral (LHb) subdivisions (Figure 4A). It receives input from medial forebrain bundle through stria medullaris and projects to the midbrain via the fasciculus retroflexus (Sutherland, 1982).

3.2 CONNECTIVITY OF LATERAL HABENULA

The LHb receives both glutamatergic and GABAergic input from the GPi, lateral hypothalamus (LH), ventral pallidum and basal forebrain (Shabel et al., 2012; Stamatakis et al., 2016). LHb consists of several subnuclei, which form medial and lateral subdivisions. The medial and lateral subdivisions are innervated by limbic system and basal ganglia mainly through the stria medullaris (Herkenham and Nauta, 1977), (Figure 4B). Primary afferents to the medial division of the LHb arise from the limbic brain: hypothalamic areas including the LHA and the lateral preoptic area (LPO) and structures of the pallidum including the bed nucleus of the stria terminalis (BST), the substantia innominata (SI) and the diagonal band nucleus (NDB) (Herkenham and Nauta, 1977; Hikosaka, 2007; Geisler and Trimble, 2008), (Figure 4B). Those regions receive either direct or indirect innervations from the cerebral cortex. The basal ganglia project to the lateral division of LHb and in particular the entopeduncular nucleus (EPN), which is innervated by the cerebral cortex via the striatum. The EPN is the non-primate homologue of the GPi. Moreover, ventral pallidum targets LHb and those inputs are densely innervated by the nucleus accumbens core (NAcc). Inputs from ascending areas like dorsal raphe (DR), the VTA, and the interfascicular and paranigral nuclei (midline region of the VTA) reach LHb through the fasciculus retroflexus. Those later projections provide the major dopaminergic inputs to habenula (Sutherland, 1982). Contrary to that, the mesohabenular neurons cannot release DA due to luck of the vescicular monoamine trasporter 2, necessary for DA packaging in the synaptic vesicles (Root et al., 2015).

Ascending projections from LHb, project primarily to brainstem structures, while less dense efferents target forebrain areas (Herkenham and Nauta, 1977; Hikosaka, 2007; Geisler and Trimble, 2008), (Figure 4B). Within the brainstem LHb targets the monoaminergic centers of VTA (dopaminergic), medial and dorsal raphe (serotonergic) as well as laterodorsal tegmentum (cholinergic). As aforementioned, LHb's main provider of dopaminergic inputs is VTA. Thus, LHb forms the crossroad between the cortical regions and brainstem monoaminergic centers. LHb is comprised mostly by glutamatergic neurons. However, their neurochemical expression patterns seem to be heterogeneous (Geisler and Trimble, 2008).

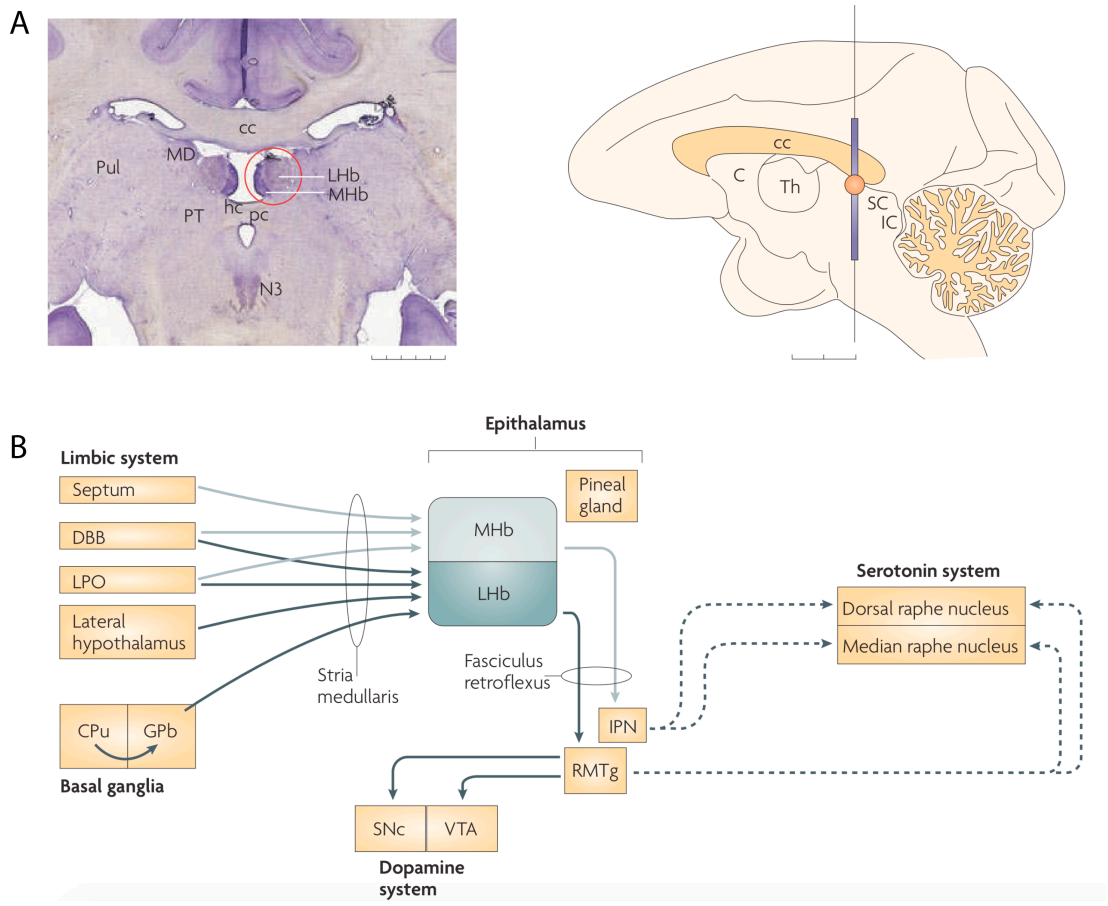

Figure 4. Neuroanatomy of habenula. A Left: Coronal brain section of a rhesus monkey, focusing on the habenular complex (context in red circle). Both medial and lateral parts of habenula are indicated. Right: Sagittal monkey brain cartoon. The purple line indicates the position of the histological section on the left in the anteroposterior axis, while the orange dot corresponds to the location of habenula. **B** Connectivity patterns of the habenular complex. Light and dark lines represent the heterogeneous connectivity patterns of the MHb and LHb respectively. Both medial and lateral parts of habenula receive input through stria medullaris and project to their target areas through the fasciculus retroflexus. The limbic system projects to the MHb, which in turn targets the interpeduncular nucleus (IPN). The IPN ultimately reaches the raphe nuclei. The LHb gets input from limbic structures as well as the basal ganglia and projects to the aminergic midbrain centers (dopaminergic, VTA and SNc; serotonergic, dorsal and medial raphe nuclei). Image from: Hikosaka 2010, Nature Reviews Neuroscience 11, 503–513.

3.3 LHB CIRCUITRY IN REWARD PREDICTION

The LHb has gained a lot of scientific attention due to its involvement in the reward prediction and aversion, which are central in goal-directed behaviors (Hikosaka et al., 2008; Zhao et al., 2015). Dysfunction of LHb has been linked to mental illness (Proulx et al., 2014) that is associated with maladaptive signaling for reward and punishment. A unique feature of LHb is its influence to the midbrain neuromodulators, serotonin and dopamine (Proulx et al., 2014). Neuronal activity in LHb is increased by aversive stimuli and decreased by unexpected rewards, with the activation preceding the inhibition of VTA dopaminergic neurons (Matsumoto and Hikosaka, 2007). These data indicated that activation of LHb exerts an inhibitory effect upon VTA dopaminergic neurons, even though LHb is a highly glutamatergic structure. In vivo activation of the glutamatergic LHb projections to VTA (Lammel et al., 2012) but interestingly also to RMTg (Stamatakis and Stuber, 2012) produces aversive response behaviors. Additionally, GABAergic RMTg projections to VTA DA neurons are activated after an aversive stimulus and inhibited in the presence of reward (Jhou et al., 2009), thus, supporting the role of LHb-RMTg-VTA circuit in aversive response.

In contrast to the reward-related behavioral implications of LHb efferents to midbrain, only the last few years, research has shed light into how modulation of projections to LHb can affect behavior. Neurons from the border region of GPi – LHA provide glutamatergic input to LHb (Shabel et al., 2012; Stamatakis et al., 2016). The LHb projecting GPi neurons, are caring both excitatory and inhibitory components (Shabel et al., 2012). These projections were hypothesized to encode negative reward prediction errors in a similar way with the LHb neurons (Hong and Hikosaka, 2008).

In response to the reward omission, GPi neuronal activation precedes the activation of LHb neurons (Hong and Hikosaka, 2008). However, it has been hard so far to experimentally discriminate among the glutamatergic subpopulations of GPi-LHA border in order to specifically target either the GPi or LHA populations. Thus, research focusing on the support of GPi-LHb mediating aversion signals is neglecting the contributions of LHA projections to LHb. In fact, recent focus on the LH-LHb glutamatergic projections revealed that inhibition of this pathway promotes reward consumption (Stamatakis et al., 2016) and reduces escape behaviors in response to aversive stimuli (Lecca et al., 2017).

3.4 PAPER III

A HYPOTHALAMUS - HABENULA CIRCUIT CONTROLS AVERSION

3.4.1 Aims and Purpose

The LHb is an evolutionary conserved structure of the epithalamus that dynamically mediates self-adaptation to the continuous and diverse input of environmental stimuli. Dysfunction of this flexible response to the new contexts and environmental input is consistent with behaviors observed in affective disorders (i.e. depression). LHb acts as a hub in conveying forebrain signals (including RPE) to the aminergic centers of the midbrain. More specifically, LHb receives input from limbic areas and basal ganglia and projects to dopaminergic neurons in VTA/SNc and to serotonergic neurons of the raphe nucleus.

The aim of this project is to delineate how the glutamatergic inputs to lateral habenula originating from the GPi-LHA border region, can differentially control reward and aversion states and ultimately affect decision-making. For this reason we aimed to reveal the neuroanatomy and molecular diversity of the GPi/LHA border region in order to identify its distinct anatomical connections with LHb and study their connectivity and behavioral implications in the reward processes.

3.4.2 Methodological approaches

All in all, the methods involved in project III included viral tracing, electrophysiology, single-neuron nuclei RNA sequencing, advanced behavior together with in vivo optogenetics and in-vivo calcium imaging.

In order to study the molecular diversity of the GPi/LHA areas we used immunohistochemistry, RNA-FISH and single-neuron nuclei RNA sequencing (snRNA seq) on a variety of transgenic mouse lines (Vglut2-Cre:TVA, Vglut2-Cre, Sst-Cre, Sst-Cre:H2bGFP, Vgat-Flpo) to specifically target the subpopulations of interest. LHb cell-type specific and LHb projection-specific whole-brain monosynaptic retrograde tracing was performed by using the Rabies tracing system.

We applied electrophysiology in combination with optogenetics to prove the GPi glutamate-GABA and Sst co-transmission in LHb versus the LHA glutamate transmission in LHb (in Vglut2-Cre and Sst-Cre mouse lines).

Optogenetics were additionally combined with classical and advanced behavior (real-time place

preference and probabilistic two-choice task) to identify the specific pathway that mediates aversive responses and to examine how the manipulations of the pathway alters the value of an outcome previously experienced, to eventually affect decision-making (Vglut2-Cre, Vglut2-Cre/Vgat-Flpo and Sst-Cre mouse lines were used).

For the last set of experiments we combined calcium imaging with fear conditioning behavioral paradigm. We aimed to delineate how the LHA glutamatergic population as well as the LH-LHb glutamatergic pathway encode and predict aversive signals (Vglut2-Cre and Vglut2-Cre/Vgat-Flpo mouse lines were used).

3.4.3 Results of Paper III

To study the molecular profile of neuronal populations and understand the function of their circuits in animal models, we first explored whole-brain glutamatergic (Vglut2) inputs to LHb. By injecting Rabies-EYFP in the LHb of Vglut2-TVA mice, we were able to map labeled cells in a variety of areas, focusing mostly at the GPi-LHA region. In order to determine the molecular identity of Vglut2 neurons in GPi versus LH, single-neuron nuclei RNA sequencing was applied (Grinberg et al., 2013), in microdissected GPi regions of Vglut2-Cre::H2bGFP reporter mice. The data analysis aimed to capture the GABA/glutamate co-releasing population of GPi (Shabel et al., 2014) and its potential coexpressed markers. Indeed, a cluster of Vglut2-Vgat-Sst co-expressing GPi neurons was identified. The anatomical distribution of this GPi population was further explored with single-molecule fluorescent in situ hybridization, which confirmed the data from snRNA-seq. This technique also revealed that the LHA population was positive only for Vglut2 but negative for Vgat and Sst. The snRNA sequencing and IHC results, were complemented by combining optogenetics and electrophysiology on the Sst GPi-LHb and the Vglut2 LH-LHb pathways. For the last part of the anatomical experiments, we explored the divergence of whole-brain inputs to Vglut2 GPi-LHb and Vglut2 LH-LHb pathways, by employing rabies tracing in Vglut2-Cre transgenic animals (Figure 5). LH-LHb and GPi-LHb pathways receive input from limbic and sensorimotor areas respectively (Figure 5G).

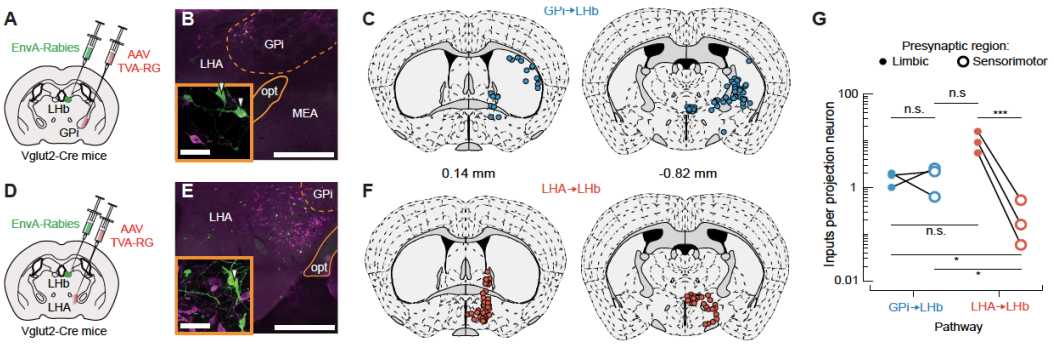

Figure 5. Whole-brain inputs to the GPi-LHb and LHA-LHb pathways. **A** Schematic of the experimental strategy for the Rabies-tracing: the Cre dependent helper virus (AAV-TVA-V5-RG) is injected in the GPi of Vglut2-Cre mice. The Rabies-GFP virus is injected in the LHb of Vglut2-Cre mice and it is being internalized by the TVA receptor found in the LHb terminals arising from the Cre expressing primary populations in GPi. Then retrograde rabies spread is performed. **B** Histological section indicating the anatomical distribution of the primary population in GPi as defined by the co-expression of the TVA-receptor (in purple) and the Rabies-GFP (in green). **C** Brain cartoon showing the basal ganglia inputs to the GPi-LHb pathway. **D** Schematic of the experimental strategy for the Rabies-tracing: the Cre dependent helper virus (AAV-TVA-V5-RG) is injected in the LHA of Vglut2-Cre mice. The Rabies-GFP virus is injected in the LHb of Vglut2-Cre mice and it is being internalized by the TVA receptor found in the LHb terminals arising from the Cre expressing primary populations in LHA. Then retrograde rabies spread is performed. **E** Histological section indicating the anatomical distribution of the primary population in LHA as defined by the co-expression of the TVA-receptor (in purple) and the Rabies-GFP (in green). **F** Brain cartoon showing the inputs from limbic structures to the LHA-LHb pathway. **G** Qualification of the whole-brain limbic versus sensorimotor inputs to either GPi-LHb or LHA-LHb pathway. GPi-LHb pathway receives input primarily from sensorimotor areas while the LHA-LHb from limbic structures. Image from: Lazaridis I. 2019, Mol Psychiatry 24(9):1351-1368.

The next part of the project aimed to define the roles of GPi-LHb and LH-LHb pathways in aversive behaviors, by using optogenetics. Initially, the Vgat/Vglut2 and Sst population of GPi, and the Vglut2 population of LHAwere optogenetically activated, while mice were performing a real-time place preference assay (Figure 6). The main finding of these experiments was that the activation of Vglut2 LH-LHb produced a strong avoidance behavior and not Vglut2 GPi-LHb, Vgat/Vglut2 GPi-LHb or Sst GPi-LHb (Figure 6D). We then explored if these pathways can bias already shaped action values in future decisions. Therefore, mice were trained in an action value task where they could alternate their decision to visit the left or right port, depending on the evaluation of the action and the reward history. During this task the Vglut2 LH-LHb or Vgat/Vglut2 GPi-LHb populations were optogeneticaly activated at the moment of choice for a port. Activation of the Vglut2 LH-LHb could decrease the value of a previously rewarding choice, leading to a switch of choice in a subsequent trial. We did not observe the same outcome when the Vgat/Vglut2 GPi-LHb pathway was activated. Another set of experiments involved in-vivo GCaMP imaging for mapping the neuronal activity of the LHA

Vglut2 population during classical behavioral conditioning paradigms for reward and punishment. The results revealed a functional heterogeneity of this population, with distinct neuronal clusters being activated either by foot-shocks or reward. The cluster of neurons responding to foot-shocks is hypothesized to participate in the LH-LHb pathway. In order to prove that, in-vivo GCaMP imaging was performed specifically on Vglut2 LH-LHb population. A retrograde HSV-Flpo-mCherry virus was used to label inputs to LHb of Vglut2-Cre mice and a CreON/FlpoON – GCaMP6 AAV vector was injected to LHA to be taken up only by the LHb projecting (Flpo), Vglut2 (Cre) neurons. Mice were then trained in a classical fear-conditioning paradigm, where a tone (condition stimulus) would predict a mild foot-shock (unconditioned stimulus). The results indicated that Vglut2 LH-LHb neurons were activated during aversive events and could also acquire prediction signals for aversion.

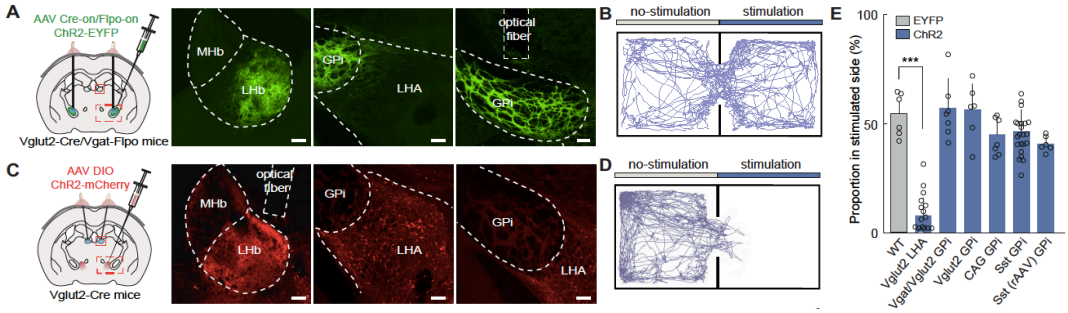

Figure 6. Optogenetic activation of the LHA-LHb glutamatergic but not of the GPi-LHb glutamatergic/GABAergic pathway, induces an avoidance response. **A** Left: Illustration of the experimental approach: ChR2 in injected in the GPi of Vglut2-Cre/Vgat-Flpo mice and the optical fiber is also implanted in the GPi. Thus, the GABA/glutamate co-expressing GPi subpopulation is targeted. Right: $GPi^{Vglut2+/Vgat+}$ axonal terminals in LHb and representations of the injection site in GPi. **B** Representative locomotion trace: a Vglut2-Cre/Vgat-Flpo mouse performing in the place preference paradigm. The mouse did not show preference for either the stimulation-paired side or for the no-stimulated side. **C A** Left: Illustration of the experimental approach: ChR2 in injected in the LHA of Vglut2-Cre mice and the optical fiber is implanted in LHb. Thus, the glutamatergic LHA-LHb pathway is targeted. Right: $LHA^{Vglut2+}$ axonal terminals in LHb and representations of the injection site in LHA. **D** Representative locomotion trace: a Vglut2-Cre mouse performing in the place preference paradigm. The mouse showed strong preference no-stimulation side indicating that the LHA^{Vglut2}-LHb pathway activation induces aversive responses. **E** Quantification of the aversive responses in a series of optogenetic activations of either axonal terminals in LHb or of GPi cell-bodies, revealed that only the LHA^{Vglut2}-LHb can induce a strong avoidance response to the stimulation. Image from: Lazaridis I. 2019, Mol Psychiatry 24(9):1351-1368.

4

THE PARABRACHIAL NUCLEUS

4.1 INTRODUCTION

The parabrachial nucleus (PB) is an evolutionary conserved area of the hindbrain that acts as hub for interoceptive and exteroceptive noxious and autonomic control-related inputs to promote appropriate behavioral and physiological responses for the survival of species (Fulwiler and Saper, 1984; Bernard and Bandler, 1988; Fuller et al., 2011; Chiang et al., 2019). PB is situated in the pons-midbrain junction and it is subdivided into a medial (PBm) and lateral (PBl) part by the superior cerebral peduncle fiber tracts (scp) that link the midbrain with the cerebellum. In rodents, the cytoarchitecture of the PBm and PBl can be further subdivided into dozens of subregions based on their molecular profiles and their functional connectivity patterns (Figure 7), (Fulwiler and Saper, 1984; Hashimoto et al., 2009). The vast majority of PB neurons is glutamatergic and expresses the marker Vglut2 (Mu at al., 2017). The PB^{Vglut2} neurons are thought to convey interoceptive signals including breathing (Yokota et al., 2015). Additionally, there is a substantial amount of neurons expressing GABA (Geerling et al., 2017). PBm is characterized by a more heterogeneous cytoarchitecture in regards to the morphological features and size of the cells, whereas PBl exerts more homogeneous characteristics that have been extensively studied over the years (Saper and Loewy, 1980; Fulwiler and Saper, 1984).

4.2 CONNECTIVITY OF PB

Studies using retrograde and anterograde tracing tools have revealed the pathways to and from the PB (Figure 7). These ascending and descending pathways are converging in PB and contribute to the regulation of autonomic control in response to sensory input. In detail, information about taste, visceral malaise, fluid intake and cardiovascular activity arise to PBl primarily from the nucleus of the solitary tract (NTS) (Herbert et al., 1990). Additional to the NTS, chemosensory inputs to PBl arise also from the rostral medulla and the Kölliker-Fuse nucleus; conveying inputs regarding respiratory control (Rosin et al., 2006; Kaur et al., 2017). Nociceptive, thermal and itch signals reach the PBl from the trigeminal and spinal dorsal horns, through the spinoparabrachial tract (Hylden et al., 1989; Cameron et al., 2015; Rodriguez et al., 2017; Barik et al., 2018; Morrison and Nakamura, 2019).

PB is characterized by reciprocal connectivity with higher brain structures, fact that is consistent with its role in conveying sensory information to forebrain in order to shape behavior and regulate autonomic function. The central amygdala (CEA), the bed nucleus of the stria terminalis (BST), and numerous hypothalamic nuclei (paraventricular, periventricular, dorsomedial, anteroventral, ventromedial, and lateral hypothalamic nuclei and the preoptic area) are reciprocally connected with the PBl. More specifically, PBl densely projects to BST and CEA, with the vast majority of inputs arising from the external part of PBl, while both PBl and PBm project to the hypothalamic nuclei (Saper et al., 1980; Fulwiler and Saper, 1984). The paraventricular and gustatory thalamic structures also receive input from the PBm and PBl subdivisions, as well as several cortical areas (infralimbic, prelimbic, insular) (Saper and Loewy, 2016; Morrison and Nakamura, 2019). PB efferents to the brainstem target reticular motor-related areas (Geerling et al., 2017; Barik et al., 2018) and the rostral medulla, which is mostly implicated in pain and thermo-regulation (Roeder et al., 2016; Chen et al., 2017; Morrison and Nakamura, 2019).

4.3 MOLECULAR SUBDIVISIONS OF PB

Describing the molecular heterogeneity of PBl, it has been shown that while some neurochemical markers (receptors, neuropeptides and transcription factors) are broadly expressed throughout PBl, there are some with discrete expression patterns only within specific subregions (Block and Hoffman, 1987; Zagami and Stifani, 2010; Miller et al., 2012). This distinct PBl neurochemistry is also marked by differential connectivity patterns, thereby enhancing the idea that the PBl subregions can be functionally segregated. Example markers that are uniquely expressed in define regions of PBl and subdivide the nucleus into its external lateral (PBle) and dorsal lateral (PBld) parts are the following: the PBle can be anatomically identified by the expression patterns of the calcitonin gene-related peptide (CGRP) (Campos et al., 2016) and tachykinin (Tac1) (Barik et al., 2018) while the PBld area expresses the oxytocin receptor (Oxtr) (Ryan et al., 2017) and cholecystokinin (Cck) (Garfield et al., 2014).

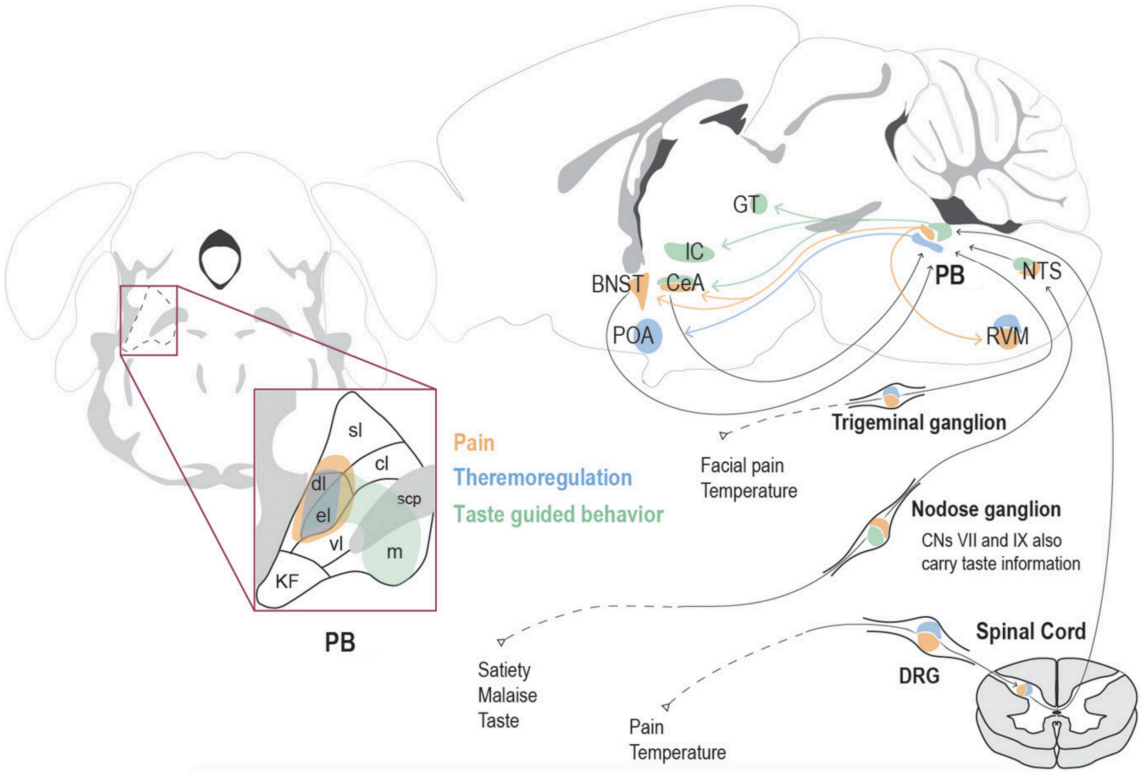

Figure 7. Neuronal networks of the parabrachial complex mediating pain, thermal-sensation and gustatory-related behavior. Left: Schematic of the main subdivisions of the parabrachial nucleus (sl, superior lateral; dl, dorsal lateral; el, external lateral; cl, central lateral; vl, ventral lateral; m, medial; KF, Kölliker Fuse nucleus). The superior cerebellar peduncle (scp) dissects the PB into its medial and lateral parts. The lateral part of the nucleus receives sensory information regarding internal physiological stimuli as well as environmental stimuli including pain, threats and temperature sensation. Right: sensory information reaches PB through primary neurons in the trigeminal ganglion, nodose ganglion or the dorsal root ganglia (DRG) of the spinal cord. Peripheral pain-related and thermal input arrive to the lateral part of PB and enhance the connections with forebrain structures such as the CEA and BST along with the preoptic area (POA) of the hypothalamus. The medial part of PB mediates gustatory signals through its connections with the gustatory thalamus. Image from Chiang, M. C. 2019, The Journal of Neuroscience, 39(42), 8225–8230.

4.3.1 The PBl^{CGRP} population encodes danger

The PBl^{CGRP} population is conveying a variety of noxious stimuli that include hypercapnia, ingestive behavior, pain and itch (Kaur et al., 2017; Han et al., 2018; Gaulrlau and Bernard 2001; Campos et al., 2018), through their connections to the lateral capsule region of CEA and the oval nucleus of BST (Carter et al., 2013). These stimuli can be life threatening (i.e. visceral and cutaneous pain as a response to deleterious food), on top of other environmental stimuli for example novel environments and fear conditioning through learning. PBl^{CGRP} neurons are coexpressing glutamatergic markers and to some extent they overlap with Tac1, neurotensin and PACAP (Sanz et al., 2009). The PB^{CGRP} population encode negative valence; when gluatmatergic release is blocked in target areas there is a decrease in behavioral responses to threatening stimuli (i.e. reduced itching in response to a pruritic stimulus, increased consumption of novel food, disrupted conditioning of avoidance in response to aversive

stimuli) (Carter et al., 2015; Han et al., 2015b; Campos et al., 2018; Chen et al., 2018; Palmiter, 2018). How this small CGRP-expressing population in PBl can mediate this diversity of behaviors remains to be further explored.

4.3.2 The PBl^{Tac1} population implications in nociception

The PBl^{Tac1} population is mainly situated within the PBle borders but Tac1 neurons are also sparsely distributed throughout the PBd (Barik et al., 2018). PBl^{Tac1} neurons are partially overlapping with the PBl^{CGRP} population and both have similar projection patterns. Chemogenetic activation of PBl^{Tac1} neurons can induce motor responses (increased jumping) to noxious heating and this response is due to PBl^{Tac1} connections with the caudal-dorsal medullary reticular formation (MdD) (Barik et al., 2018).

4.3.3 The PBl^{Oxtr} population and fluid intake

The PBl^{Oxtr} neurons are mediating information regarding fluid intake through inputs from areas also known to be involved in drinking behavior: excitatory input from the oxytocin-expressing population in the paraventricular nucleus, the Cck-expressing neurons in NTS as well as other glutamatergic NTS neurons (Ryan et al., 2017). Chemogenetic activation of PBl^{Oxtr} population supresses water consumption in thirsty mice, but no effect is observed in food consumption (Ryan et al., 2017).

4.3.4 The PBl^{Cck} population and glucose homeostasis

The PBl^{Cck} population belongs to the central circuits involved in glucose homeostasis (Garfield et al., 2014). The majority of Cck-expressing PBl neurons are situated in the dorsal divisions and co express the leptin receptor (Flak et al., 2014). However, some Cck-neurons are scattered in the PBl as well. PBl^{Cck} neurons mediate noxious sensory inputs such as starvation and hypoglycemia and a counter-regulatory response is initiated through connections with the steroidogenic-factor 1 (SF1)-expressing neurons of the ventromedial nucleus of the hypothalamus (VMH) (Garfield et al., 2014). The PBl^{Cck} – VMH^{SF1} microcircuit is important in mediating hepatic glucose production as a response to hypoglycemia. Therefore, the PBl^{Cck} population is critical for the homeostatic function in health and is probably implicated in glucose-related disease states, for example, diabetes.

4.4 PAPER IV

CHARACTERIZATION OF A CHOLINERGIC-GLUTAMATERGIC POPULATION IN THE LATERAL PARABRACHIAL NUCLEUS

4.4.1 Aims and Purpose

The PB acts as a hub in mediating noxious sensory information to forebrain structures in order to adjust behaviors and modulate innate physiological responses. The lateral part of PB can be divided in numerous subregions based on the distinct neurochemistry and connectivity of the area. The molecular diversity of the PBle can underlie the pallet of sensory modalities it mediates to ultimately promote the well being of individual organisms.

In this direction the main goals of project IV were to investigate the molecular diversity of the neuronal populations lying in the PBl and examine their differential connectivity patterns. The molecular markers explored here included the Vglut2, the choline acetyltransferase (Chat), Oprm1 and cholecystokinin (Cck). Thus, we aimed for a finer neuroanatomical dissection of the nucleus.

4.4.2 Methodological approaches

For this project we used a variety of transgenic mouse lines including Vglut2-Cre, Chat-Cre, Oprm1-Cre and Cck-cre to target the respective neuronal populations of the PB. First we explored the potential overlap among these populations by coupling immunohistochemistry with the genetic labeling of each population (the AAV- ChR2-mCherry was injected in PB). Anterograde tracing was performed by using optogenetic tools, where the membrane-bound protein ChR2-mCherry allowed the identification of the $PBle^{Chat}$, $PBle^{Oprm1}$ and $PBld^{Cck}$ neuronal axons and axonal terminals. The rabies monosynaptic retrograde tracing was performed to study the whole-brain inputs to the Chat and Oprm1 populations in PBle. Last, an experiment of retrograde tracing with retrobeads in known input regions to PBle (CEA and BST), allowed for the identification of neurons within PBl, sending collaterals to both of these areas.

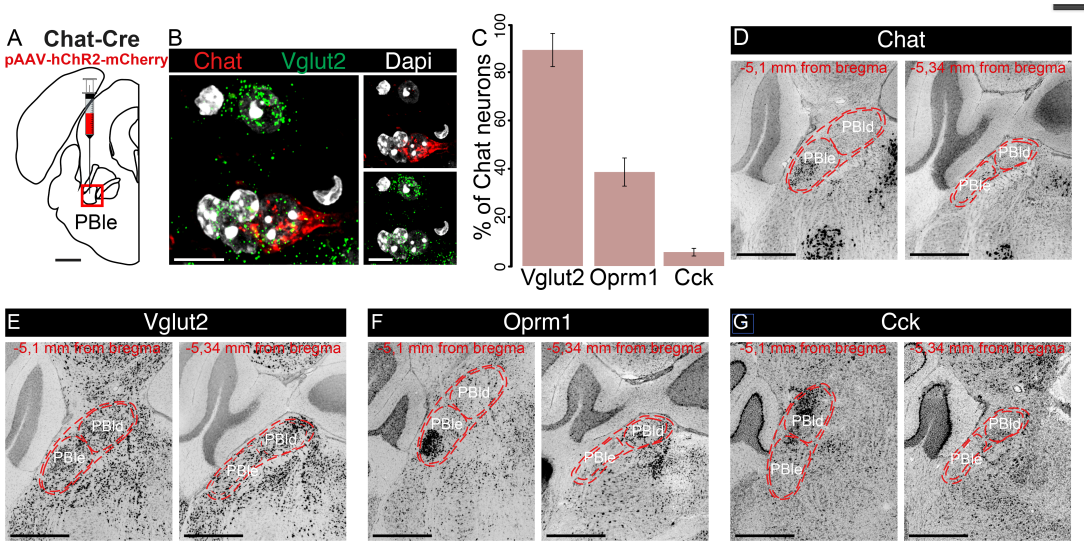

Figure 8. The molecular dissection of PBl. A Schematic representing the genetic labeling of the cholinergic PBle population: the Cre-dependent virus expressing ChR2-mCherry was injected in the PBl of a Chat-Cre mouse line. **B** The cholinergic population of PBle co-expresses the glutamatergic marker Vglut2 and **C** partially Oprm1, but there is almost no overlap with the Cck. **D-E-F-G** The differential expression of the markers Chat, Vglut2, Oprm1 and Cck in two A-P coordinates (-5,1 mm and -5,34mm from bregma on the left and right respectively; images from Allen Brain In situ experiments). Vglut2 is expressed throughout the dorsal-ventral axis of PB. Chat and Oprm1 neuronal distribution is mainly restricted in the external part of PBl while the Cck population is mostly lying in the dorsal part of the nucleus. Image from: Tzortzi O. 2020, unpublished data.

4.4.3 Results of Paper IV

We identified and described two unexplored neuronal populations of the PBle. These populations express either choline acetytransferase or the μ opioid receptor (Figure 8D, 8F). We further explored a population of the PBld expressing cholecystokinin (Figure 8G).

The PBle^{Oprm1} population is larger than the PBle^{Chat}, even though both populations are occupying the same territory in PBle. Thus, the cholinergic population can more specifically define the external lateral subregion of the PBl. Moreover, the PBle^{Chat} neurons are glutamatergic and partially express Oprm1 (Figure 8B, 8C). However, the Cck neurons are found in the dorsal part of PBl adjacent to the PBle and do not exhibit overlap with the PBle^{Chat} neurons (Figure 8C). Neurochemical markers found in the Oprm1 and Chat PBle area include among others the CGRP and Tac1 expressing populations, which are also glutamatergic (Campos et al., 2016, Barik et al., 2018). According to the literature, the PBle^{CGRP} and PBle^{Tac1} neurons rely sensory information regarding threat memory and pain related to noxious heat, to other forebrain structures. Such sensory inputs involve reciprocal connectivity of forebrain structures with the PBl.

We aimed to study the reciprocal connectivity of the PBle^{Chat} and PBle^{Oprm1} populations. Additionally, we wanted to compare and contrast the differential projection patterns of PBle and PBld. Therefore, anterograde and retrograde tracing was applied in the PBl.

The monosynaptic retrograde rabies virus tracing revealed the whole-brain presynaptic inputs to PBle^{Chat} and PBle^{Oprm1} neurons (Figure 9A). Despite their close proximity, these populations get afferents from overlapping as well as non-overlapping areas. The largest number of ascending projections to both PBle^{Chat} and PBle^{Oprm1} populations arise from the CEA and BST nuclei (Figure 9B, 9D). Other common input structures included hypothalamic areas (LHA), the superior colliculus (SC) and areas of the midbrain reticular nucleus (MRN). However, only the PBle^{Oprm1} received input from thalamic nuclei (VAL, VM) and also non-dopaminergic input from the SN.

Anterograde tracing by using the membrane-bound ChR2-mCherry allowed for the identification of neuronal axons and axonal-terminals in the various PBN targets. The results revealed that both PBle^{Chat} and PBle^{Oprm1} populations send projections to the CEA and BST, which in combination with the retrograde tracing data, confirmed that there is reciprocal connectivity between PBle-CEA and PBle-BST for either of the PBle populations. The PBld^{Cck} also exhibited efferent connectivity to CEA and BST. These results are in accordance with the well-established knowledge of PBl mediating fear and pain related stimuli to forebrain structures including the CEA and BST (Campos et al., 2016; Barik et al., 2018). Additional outputs for the PBle^{Oprm1} neurons were the parafascicular nucleus (PF), the periaqueductal gray matter (PAG) and zona inserta (Zi), while the PBld^{Cck} population also projected to MRN, PAG and several thalamic nuclei.

For the last part of the project we examined weather the PBl input to CEA and BST, arise from overlapping or distinct neuronal populations of PBl. Therefore, dual targeting of CEA and BST with retrograde tracer was performed. The results revealed that overlapping as well as distinct groups of PBl neurons project to CEA and BST.

Overall, we have identified a neuronal subtype in PBl, which is characterized by dual co-transmission of acetylcholine and glutamate. We propose that cholinergic markers expressed by PBl neurons can sufficiently define the external subdivision of PBl, while the Cck expressing population will more specifically target the dorsal aspects of PBl. The PBl nucleus in its whole is characterized by reciprocal connectivity with the CEA and BST forebrain structures. Therefore, this type of connectivity cannot be used for the anatomical dissection of the external and dorsal subdivisions of PBl. The fact that some of the PBl neurons preferentially target either the CEA or BST and some send collaterals to both CEA and BST, adds an extra level of complexity to the system, suggesting that these distinct projection patterns form distinct neuronal networks. The matter of reciprocity between the CEA and BST areas with the PBl we hypothesize it is a feedback network from CEA and BST regulating the sensory information the PBl conveys to the CEA and BST. Advances in mouse genetics can now aid the fine dissection and discrimination of the PBl subnuclei based on their molecular characteristics, in order to further identify the specific behaviors they mediate.

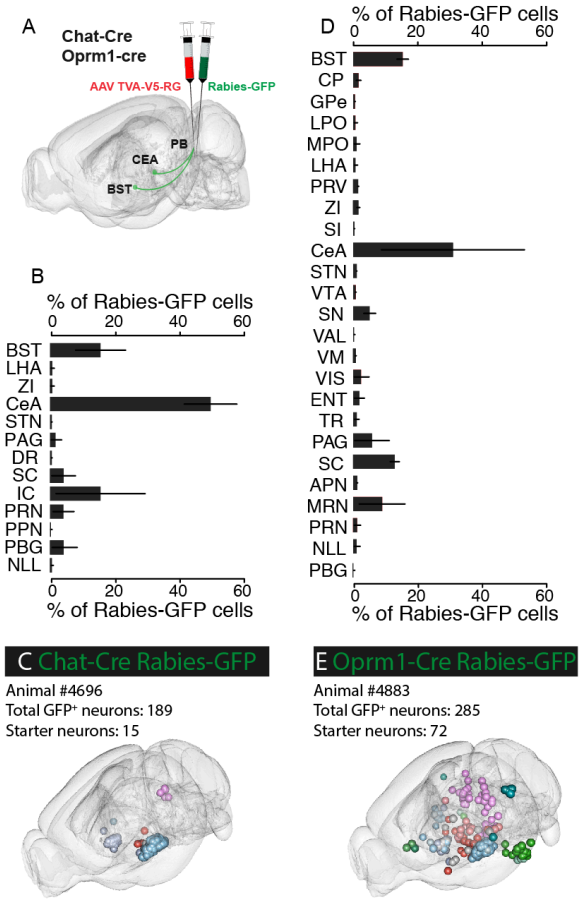

Figure 9. Whole-brain inputs to the PBle^{Chat} and PBle^{Oprm1} populations. A retrograde rabies tracing. A Schematic of the experimental strategy for the Rabies-tracing: the Cre dependent helper virus (AAV-TVA-V5-RG) is injected in the PB of either Chat-Cre or Oprm1-Cre mouse lines. The Rabies-GFP virus is also injected in the PB of either Chat-Cre or Oprm1-Cre mouse lines and it is being internalized by the TVA receptor in the Cre expressing primary populations and then retrograde rabies spread is performed. **B** Whole-brain inputs to **PBle^{Chat}.** The main forebrain structures projecting to **PBle^{Chat}** include: the CEA, BST, IC and several pontine nuclei. **C** Brain cartoon representing the whole-brain GFP input signal (189 neurons) to the **PBle^{Chat}** population (15 starter cells). **D** Whole-brain inputs to **PBle^{Oprm1}.** The main forebrain structures projecting to **PBle^{Oprm1}** include: the CEA, BST, PAG, SC, SN and the reticular formation. **E** Brain cartoon representing the whole-brain GFP input signal (285 neurons) to the **PBle^{Oprm1}** population (72 starter cells). Image from: Tzortzi O., 2020, unpublished data.

CONCLUSIONS

The interest in neurocircuits that mediate rewarding and anti-rewarding processes goes back decades and it was triggered by observations where animals would work to get food, sugar, addictive drugs etc. and even to activate brain areas or pathways. On the contrary, animals would also work to avoid harmful situations or to stop the activation of pathways mediating aversive processes. Behavioral and physiological responses that would maximize exposure to reward or minimize exposure to danger and distress are characterized by complex neurochemistry and brain network connectivity. In this direction, new technologies and advances in mouse genetics have allowed for the cell-type-specific and projection-specific targeting so that experimental efforts can interfere the molecular and anatomical complexity of networks.

The main interests of this thesis regard to the brain reward and aversion circuits and how they mediate motivated behaviors. **Chapters 1 and 2**, extensively describe the anatomy and functions of basal ganglia, as part of the reward system. The basal ganglia are mediating a great variety of reward-related and motivated behaviors, with the striatum being the key structure in these processes. Here we have worked towards the direction of developing a flexible framework (**Paper I**) that can be easily used to handle a variety of anatomical data (cell-bodies, axonal processes, dendrites) produced by a variety of experimental approaches ranking from tracing studies, immunohistochemistry and in situ hybridization. This is an open-access tool that can be used to integrate whole-brain information on a personalized brain map. It is crucial for scientists to establish common neuroanatomical maps with information about the identities, topography and connectivity patterns of neurons involved in a variety of behavioral aspects. In this way, the various experimental approaches could lead to reproducible results.

In **Paper II**, we identified striatal units of distinct molecular identities and unique topographic characteristics based on sn-RNA-seq and in situ data. First, we molecularly dissected the patch, exopatch and matrix compartments of the striatum (patch markers: Oprm1+ /Sema5b+/ Id4-; exopatch markers: Oprm1+ /Sema5b-/Id4+; matrix markers: Oprm1- /Sema5b-/Id4+). Thus, the combinatorial presence or absence of the Oprm1/Sema5b/Id4 markers can be used to specifically define the striatal subregions and subpopulations of interest. For example, by generating the appropriate transgenic mouse (i.e. in our case Oprm1-Cre:Id4-Flp), it will be the first time that the true behavioral contributions of the exopatch could be studied. We have also found an unexplored D1+ MSN subpopulation expressing Col11a1 and Sema5b but not Oprm1. Based on the classic literature only the patch Oprm1-expressing and not the matrix neurons project to SNc. It is interesting that this Col11a1+/Oprm1- population also projects to SNc. It remains to see in the future what functional processes it mediates. Last, we molecularly characterized the cortex-targeted striatal subregions. The corticostriatal inputs divide striatum into four quadrants, the borders of which are defined by the dorsoventral and mediolateral axes of striatum and mediate distinct functional processes (Hintiryan et al., 2016). We anatomically visualized this projection pattern based on the expression of the markers Crym, Dlk1 and Grp155 in the dorsomedial, ventromedial and lateral aspects of striatum respectively. Thereby,

we have created a spatial and molecular map of striatum that can be used to study the striatal implications in the motivational processes, drug abuse, motoric activity etc.

Studies in motivational behaviors reveal its influences by fluctuations in the aminergic activity (i.e. dopaminergic, serotonergic). Thus, modulators of the aminergic systems are central in mediating rewarding and anti-rewarding processes. Lateral habenula is an evolutionary conserved epithalamic nucleus implicated in the pathophysiology of several affective disorders including depression (**Chapter 3**). LHb conveys aversive signals from its basal ganglia and hypothalamic inputs to the midbrain dopaminergic and serotonergic systems (Lammel et al., 2012). **Paper III**, revealed that the anti-reward signaling of LHb arises from excitatory hypothalamic inputs and not from GPi (basal ganglia) input. During this project, retrograde-rabies tracing revealed that the limbic brain preferentially projects to the LHA-LHb pathway, while the GPi-LHb circuit gets information from sensorimotor areas. The tracing study could explain the results from the optogenetic experiments where only the activation of the LHA^{Vglut2}-LHb and not the GPi^{Vglut2}-LHb, could induce a strong aversive phenotype when mice performed in real-time place preference. This activation could also reduce the value of a previously rewarding action. Thus, the LHA^{Vglut2}-LHb pathway can influence decision-making based on the current value of a previously experienced action. Calcium imaging in the LHb-projecting LHA^{Vglut2} population while mice performed in fear-conditioning with mild foot-shocks (US) paired with a tone (CS) showed that distinct clusters of LHA^{Vglut2} neurons were responding to the tone or the foot-shock. These results indicated that the hypothalamic input to LHb participates in Pavlovian learning, can acquire prediction signals for aversion and also encode aversive and painful stimuli.

An other hub for mediating noxious and painful internal and external information is the lateral parabrachial nucleus of the pons-midbrain boundary (**Chapter 4**). PBl gets activated and conveys nociceptive signals from the brainstem to forebrain structures. The nucleus is subdivided into more than a dozen subregions characterized by distinct expression of neurochemical markers. In **Paper IV** we have identified an unexplored cholinergic population of the external lateral part of the PBl which co-expesses glutamate. The cholonergic-glutamatergic PBle subpopulation is characterized by reciprocal connectivity with the areas of CEA and BST indicating that there is a feedback loop from the forebrain regulating the sensory information these neurons mediate. We observed that the $PBle^{Chat}$ population exhibits no overlap with the adjascent $PBld^{Cck}$. Therefore, we propose that the markers Chat and Cck can be used to separate PBl into its external-lateral and dorsal aspects respectivelly, allowing for the specific targeting of the subregions. It was recently shown that while the PBle is classicly activated by noxious stimuli, the PBld metiates rewarding signals (Han et al., 2018). However, the molecular identinty of the neurons implicated in these processes is still unknown. How the plethora of PBl molecular and spatial subdivisions are involved in reward-related behaviors is yet to be explored.

In conclusion, this thesis aims to inspire concepts on how we should preceive the complexity of neuroanatomical circuits to explain motivated behaviors. It is always important to study the brain on a multilevel scale, for I believe it will aid future scientists to have a more spherical view when attempting to disentangle the neuropathophysiologies of affective disorders.

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Those who know me have seen my care for children. All children! The children I left behind, the children I met on the way, the ones I will meet in the future: ***Olimbia, Martha, Katerina, Michalis, Leonidas, Sotiris, Sofia, Illiana, Politimi, Alexandros, Ethan, Vibeke, Nike, Oliver, Mira, Theo, Anny****.*

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Thank you all for reading my thesis.

My journey back to Ithaca continues. See you later!

Με αγάπη,

Ράνια Τζώρτζη

Στοκχόλμη, 7 Ιανουαρίου 2020

"The smile of the Child" (Το Χαμόγελο του Παιδιού) is a Greek organization that aims to help children in need: abused, living in poverty, having health issues, missing children etc. You can find more information about the organization in https://www.hamogelo.gr/gr/en/. European help line for children: **116111**.

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