Structure and function of the circuits underlying reward.

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Structure and function of the circuits underlying reward.

Δομή και λειτουργία των κυκλωμάτων που υπόκεινται της ανταμοιβής.

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Prologue

“So she was wondering in her own mind […], whether the pleasure of making a daisy-chain would be worth the trouble of getting up and picking the daisies…”

Alice in “Alice’s Adventures in Wonderland,” Carroll, (1865, p. 11)

Most of the times, effort is experienced as a burden. Yet, we tend to expend effort in order to reach a desired goal. Theories regarding motivational choice focus mostly on how the value of the goal and the strength of the reinforcement influence and explain behavior. But less is known concerning effort-based decision-making in terms of integrating the cost of an action and the value of the goal in this theory.

In a world with hidden rewards and dangers, it is a matter of survival to choose the appropriate behaviors. The optimal decision-making and the mapping of situations to actions are learned through a trial-and-error way in order to maximize reward and minimize punishment. However, limitations like delayed deliveries of reward or punishment surround and affect reinforcement learning. Who wouldn’t choose 100$ today over 1000$ in a year from now?

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Rania Tzortzi,

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Abstract

Lateral habenula (LHb) is in a unique position to process reward-related signals into proper behavioral responses, particularly during aversive, stressful situations. Its inhibitory effects upon dopamine rich areas, indicate the key role of LHb in the modulation of brain reward system. Excitatory inputs from LHb target GABAergic interneurons of both ventral tegmental area (VTA) and rostromedial tegmental nucleus (RMTg) and drive strong inhibition on dopaminergic neurons of VTA. These reward-related signals are provided to LHb from distinct populations arising from Globus Pallidus internus (GPi). However, the nature and potential regulation of the major input to LHb originating from the basal ganglia, is poorly understood. Here by using a dual viral combination of an AAV helper virus and a genetically modified rabies virus that displays specific transynaptic retrograde spread (1) we are providing anatomical evidence for a strong innervation of LHb, by $VGluT2^{+}$ glutamatergic and $VGaT^{+}$ GABAergic GPi neurons. (2) We also demonstrate that the former $VGluT2^{+}$ LHb projecting GPi neurons receive direct monosynaptic inputs arising from the matrix and patch compartments of the caudate putamen (CPu) as well as the prototypic neurons of the Globus Pallidus externus. (3) Furthermore, we show that the optogenetic excitation of the $VGluT2^{+}$ LHb projecting GPi inputs promotes a strong aversive behavior. Taken the importance of LHb as a modulatory nucleus of the dopaminergic system, the dissection of its connectivity and function will give valuable insights in the understanding of both reward-seeking behavior and depressive disorders.

Περίληψη

Η περιοχή της πλευρικής ηνίας (ΠΗ) είναι μία περιοχή στον εγκέφαλο η οποία παίζει ιδιαίτερο ρόλο στη μετατροπή σημάτων σχετιζόμενων με την ανταμοιβή σε μία συγκεκριμένη συμπεριφορά, κυρίως όσον αφορά σε δυσάρεστα και απωθητικά ερεθίσματα. Ο ανασταλτικός ρόλος που έχει στη νευρωνική λειτουργία περιοχών πλούσιων σε ντοπαμίνη καταδεικνύουν το ρόλο κλειδί που κατέχει στη ρύθμιση του κυκλώματος ανταμοιβής στον εγκέφαλο. Διεγερτικές είσοδοι από τη ΠΗ έχουν σα στόχο GABAεργικούς ενδονευρώνες την κοιλιακής καλυπτικής περιπχής (ΚΚΠ ή VTA) και της έσω ραμφοειδούς περιοχής της καλύπτρας (RMTg) και οδηγουν εν τέλει σε ισχυρή αναστολή της έκκρισης ντοπαμίνης στη ΚΚΠ. Αυτά με τη σειρά τους αποτελούν σήματα σχετιζόμενα με την ανταμοιβή που φθάνουν στη περιοχή της ΠΗ από ξεχωριστούς πλυθησμούς προερχόμενων απο την έσω ωχρά σφαίρα (GPi). Παρολ'αυτά ο φυσιολογικός ρόλος καθώς και η πιθανή ρύθμιση των κύριων εισόδων στην ΠΗ δεν είναι πλήρως κατανοητά. Σε αυτή τη μελέτη χρησιμοποιήθηκε ένας συνδυασμός ιών : ενός αδενοσυσχετιζόμενου ιού και ενός γενετικά τροποποιημένου ιού της λύσας που έχει την ιδιότητα να μεταφέρεται ανάδρομα διαμέσου των συνάψεων. (1) Χρησιμοποιώντας τον συνδυασμό ιών αποδεικνύουμε ανατομικά ότι η LHb δέχεται έντονη εννεύρωση από VGluT2^{+} (γλουταματεργικά) και VGaT^{+} GABAεργικούς νευρώνες του GPi . (2) Επιπλέον δείξαμε ότι οι παραπάνω VGluT2^{+} νευρώνες απο το GPi δέχονται μονοσυναπτικές συνδέσεις απο κύτταρα των περιοχών πλάκα-στρώμα (patch-matrix) του ραβδωτού σώματος (κερκοφόρος πυρήνας και κέλυφος) καθώς επίσης και απο τους πρωτοτυπικούς νευρώνες που βρίσκονται στην έξω ωχρά σφαίρα. (3) Τέλος, η οπτογενετική διέγερσή VGluT2^{+} εισόδων απο το GPi στην ΠΗ προκαλεί μια έντονη συμπεριφορά αποστροφής. Με δεδομένη τη σημασία της πλευρικής ηνίας σαν ρυθμιστικό πυρήνα του ντοπαμινεργικού συστήματας η αποσαφήνηση της ακριβής συνδεσμολογίας και του λειτουργικού ρόλου αυτής θα μας βοηθήσει να καταλάβουμε συμπεριφορές κινητοποίησης εξαρτώμενες απο την ανταμοιβή καθώς και ψυχικές διαταραχές όπως η κατάθλιψη.

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Abbreviations

AAV	Adeno assosiated virus
ChR2	Channelrhodopsin 2
CPP	Condition place preference
CPu	Caudate and putamen
CS	Conditioned Stimulus
DA	Dopamine
DBB	Diagonal band of Broca
DDS	Dosral dienchephalic conduction system
DRN	Dorsal raphe nucleus
EPN	Entopeduncular nucleus
GABA	γ-Aminobutyric acid
GP	Glycoprotein G
GPb	Border region of the globus pallidus
GPe	Globus pallidus externus
GPi	Globus pallidus internus
hc	Habenular commissure
IPN	Interpeduncular nucleus
LHA	Lateral hypothalamic area
LHb	Lateral Habenula
LPO	Lateral preoptic area
MD	Mediodorsal nucleus of the thalamus
MRN	Median raphe nucleus
MSN	Medium spiny neurons
N3	Oculomotor nucleus
MHb	Medial Habenula
NAcc	Nucleus accumbens core
NpHR	Halorhodopsin
pc	Posterior commissure.
PT	Pretectum
PV	Parvalbumin
RG	Rabies glycoprotein G
RMTg	Rostromedial tegmental nucleus
SNc	Substantia nigra pars compacta
STN	Subthaamic nucleus
TVA	Avian tumor virus receptor
US	Unconditioned Stimulus
V5	Protein tag
VGaT	Vesicular GABA transporter
VGluT2	Vesicular glutamate transporter 2
VP	Ventral pallidum
VTA	Ventral tegmental area

Chapter1. Introduction

1.1 Historical overview of classical conditioning

In the mid-20th century, Ivan Pavlov was the first who introduced classical conditioning paradigms with his experiments conducted on dogs. Pavlov observed that an innate biological response evoked by a potent biological stimulus could also be expressed when a neutral stimulus was provided to the animals. This was achieved after pairing the unconditioned biological stimulus (US) with the neutral one. According to classical conditioning the values of the stimuli are learned through experience and the reinforcement learning itself is based on reward.

Moving one step forward in the animal learning theories, in 1972 Rescorla and Wagner introduced a mathematical model of learning. In this model they claimed that learning is achieved when the rewarding events are not predicted or when the reward acquired is of a higher reward value than expected. The change of the value of the event is proportional to the difference between the actual and predicted outcome (prediction error) (Figure 1). The Rescorla-Wagner model of learning is a relatively simple yet powerful model of Pavlovian conditioning. Ever since, the reward prediction error hypothesis has been regarded as one of the largest achievements of computational neuroscience. This hypothesis has become the standard model for explaining reward-based learning and midbrain dopaminergic activity.

Figure 1. The Rescorla-Wagner equation. The amount of learning depends on the amount of surprise. (ΔV: change in the predictive value of stimulus V, λ: what actually happened, ΣV: what was expected). Conventionally, λ is set to a value of 1 when the US is present, and 0 when it is absent. The terms α and β, refer to the salience of the conditioned stimulus (CS) and the speed of learning for a given US respectively. Parameters α and β affect the rate of learning, but neither of them changes during learning.

1.2 Brain Reward Circuit

The brain reward system has a key role in the reinforcement of behaviors that are rewarding and the prevention of behaviors that lead to punishment. The reward system is a complex circuit composed of inter-connected regions, which involve the forebrain limbic system and its links to the midbrain (i.e. dopaminergic and serotonergic) centers (Russo and Nestler, 2013). The main dopaminergic pathways include the nigrostiatal pathway from substantia nigra pars compacta (SNc), which targets the dorsal striatum and the dopaminergic pathway from the ventral tegmental area (VTA), which targets limbic structures such as the ventral striatum, cortical areas and the amygdala.

Dopamine neurons of the VTA play a central role in the modulation of associative learning and action preparation (Schultz, 2007). Several lines of evidence indicate the importance of inhibiting the firing pattern of DA neurons. This inhibition is hypothesized to underlie behavior associated with negative reward prediction errors (Schultz, 2007) or the learning in response to aversive stimuli (Ungless et al., 2004). Phasic excitation of DA neurons is caused by rewarding stimuli and cues that predict them, while aversive stimuli and reward omission lead to the phasic inhibition of DA neurons. (Cohen et al., 2012; Matsumoto and Hikosaka, 2007; Pan et al., 2005; Schultz et al., 1997; Tobler et al., 2005; Ungless et al., 2004). Furthermore, in response to salient stimuli the firing rate of DA neurons increases and phasic dopamine is released in the ventral striatum, more specifically in the nucleus accumbens (NAc). This signaling is crucial for the initiation of motivated behaviors (Day et al., 2007; Oleson et al., 2012; Phillips et al., 2003; Stuber et al., 2008).

1.3 Reward prediction error

Dopamine is thought to have a multidimensional role in several neurobiological systems ranging from control of movement, motivation and reward-based learning, substance abuse, attention and mood regulation and has been implicated in the pathogenesis of diseases like schizophrenia, Parkinson's disease, attention deficit hyperactivity disorder (ADHD) and addiction. A lot of work has been done wordwide in order to better understand the types of

information dopaminergic neurons carry, especially regarding their implications in the reward prediction error hypothesis.

In 1998, a series of experiments conducted in monkeys by Schultz and coworkers, proposed that the phasic activity of midbrain dopamine neurons is reflecting reward prediction errors. They showed that dopaminergic neurons were activated in the occurrence of unpredicted rewards as well as in the presence of cues predicting the reward but not in the delivery of the reward as it occurred according to the prediction. However, if the reward did not come as predicted, then the activity of dopamine neurons was depressed exactly at the time when the reward would have occurred (Schultz *et.al.,* 1997). Those data indicated that dopaminergic neurons are sensitive to the magnitude of reward, the temporal occurrence as well as the presence of the reward per se (Figure 2). In other words, phasic activity of midbrain dopaminergic neurons signals a discrepancy between the reward prediction and the current reward experience (reward prediction error).

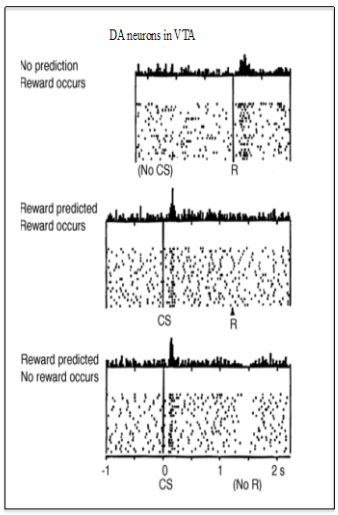

Figure 2. Reward prediction error response of a single dopamine neuron. (Top) In the absence of prediction, a reward is delivered to the subject (positive reward prediction error) and the dopamine neuron is firing right after the delivery of reward. (Middle) After training, the CS is coupled with the reward, hence the reward occurs according to the prediction of the CS (reward prediction error = 0). The firing of the dopamine neuron starts after the reward-predicting stimulus while this activation is not present when the actual reward is delivered (right). (Bottom) After training, the reward fails to occur due to a wrong behavioral response of the subject, even though the CS is present and still predicts reward (negative reward prediction error). The firing of the dopamine neuron starts after the presence of the CS but this activation is depressed exactly when the reward would have occurred, thus, indicating an internal representation of the time the reward is delivered. Image borrowed from: Schultz W, Science 275: 1593-1599, 1997

1.4 Habenula

One of the pathways connecting the limbic forebrain with the midbrain monoaminergic areas is the dosral dienchephalic conduction system (DDC), which consists of three core components: the habenular complex, stria medullaris and the fasciculus retroflexus. DDC is thought to interconnect the limbic and striatal forebrain with mid- and hindbrain areas and the point of convergence in this system is the habenular nucleus (Sutherland 1982). Habenula receives input from medial forebrain bundle through stria medullaris and projects to reach the midbrain via the fasciculus retroflexus (Sutherland 1982).

Since limbic activity is associated with emotional behaviors and reinforcing events and the basal ganglia areas are involved in the execution of movements, such point of convergence has a prominent role in the control of motivated movement processes.

1.4.1 Habenular complex

The habenular complex is localized within the epithalamus and is attached on either side of the third ventricle bilaterally (Figure 3.) (Klemm 2004). Based on cytoarchitectonic differences within the structure, Nissl proposed, in 1889, the view that the habenula is not homogenous. It is rather comprised by two closely attached and yet distinct nuclei; the cell-dense medial habenula and the lateral habenula (LHb), in which the cell population is more loosely arranged (Hermann Andres, 1999). Medial and lateral habenular complex differ a lot regarding their neuronal connections and the interactions between each other are very sparse if any. Both complexes and especially the LHb are thought to be involved in a variety of biological functions ranging from maternal behavior, pain and homeostatic processes to motivational control of behavior (Hermann Andres, 1999).

In the current thesis we focus mostly on the functions of LHb and particularly on its afferent and efferent projections that underlie motivated behaviors.

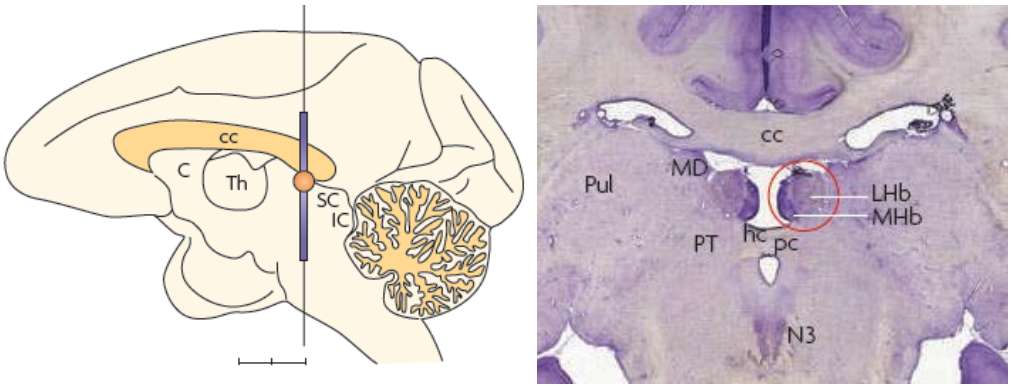

Figure 3. The habenular complex in the rhesus monkey. (Left) The monkey's brain viewed from the mesial side. C: caudate nucleus, Th: Thalamus, SC: superior colliculus, IC: inferior colliculus. (Right) A coronal histological section showing the habenula (red circle). The medially located dark region corresponds to the medial habenula (MHb), while the lateral part corresponds to the lateral habenula (LHb). The vertical extent of this section corresponds to a violet line in the left figure. MD: mediodorsal nucleus of the thalamus, Pul: pulvinar, PT: pretectum, N3: oculomotor nucleus, hc: habenular commissure, pc: posterior commissure. Image borrowed from: Hikosaka J Neurosci 28: 11825-9, 2008.

1.4.2 Lateral Habenula: Afferent connectivity

The lateral habenula consists of several subnuclei, which form medial and lateral subdivisions inside the nucleus. The medial and lateral subdivisions are innervated by two parallel circuits arising from the limbic system or basal ganglia and arborize to either medial or lateral part of LHb. Primary afferents to the medial division of the LHb arise from limbic brain regions (Figure 4): the lateral hypothalamic and lateral preoptic areas, basal forebrain structures including the ventral pallidum, substantia innominata, and diagonal band of Broca, and parts of the extended amygdala, including the bed nucleus of the stria terminalis (Herkenham and Nauta, 1977; Hikosaka, 2007a; Geisler and Trimble, 2008). Those regions receive either direct or indirect innervations from the cerebral cortex.

The lateral division of LHb receives input mainly from the basal ganglia 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 internal segment of the globus pallidus (GPi) (part of basal ganglia). Moreover, pallidal inputs from ventral basal ganglia loops (ventral pallidum) also arborize to 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 (Figure 4). Those later projections provide the major dopaminergic inputs to habenula (Sutherland, 1982).

Thus, the unique afferent connectivity of the LHb, renders this nucleus a point of convergence for limbic efferents (septum, lateral preotic, and lateral hypothalamic fibers) with striatal efferents (entopeduncular fibers and fibers from the ventral pallidum).

1.4.3 Lateral Habenula: Efferent connectivity

Ascending projections from LHb, project primarily in brainstem structures, while less dense efferents target forebrain areas (Herkenham and Nauta, 1977; Hikosaka, 2007a; Geisler and Trimble, 2008). Within the brainstem LHb targets the monoaminergic centers of VTA (dopaminergic), medial and dorsal raphe (serotonergic) as well as laterodorsal tegmentum (cholinergic), (Figure 4). 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). Additionally LHb exerts an inhibitory influence upon the dopaminergic and serotonergic centers of the brainstem (Wang and Aghajanian, 1977; Shepard *et.al.,* 2006; Matsumoto and Hikosaka, 2007. Nevertheless the hypothesis that LHb indirectly downregulates VTA dopaminergic neurons through local intermediate GABA cells, has not been proven yet (Bell *et.al.,* 2007). Yet, additional investigation is necessary in order for this hypothesis to be ruled out. Whether LHb's inhibitory effect is mediated through projections on GABAergic cells extrinsic to VTA- as well as to the rest of the monoaminergic areas- still remains to be

explored.

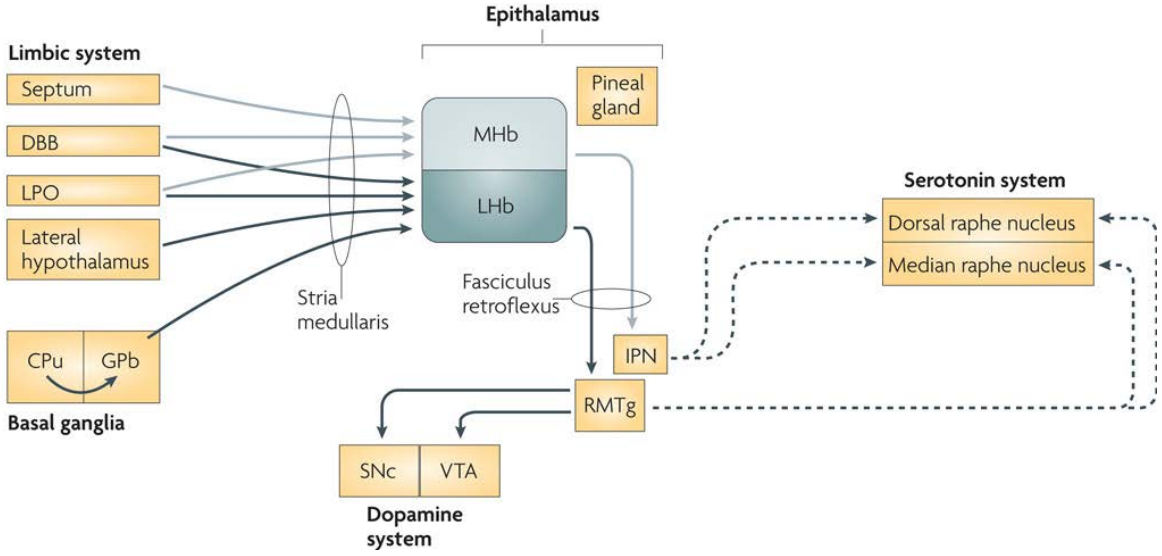

Figure 4. Afferent and efferent connectivity of habenula. The MHb receives inputs through stria medullaris from limbic areas and projects to the IPN, which in turn projects to the raphe nuclei. The LHb afferents arise mainly from the basal ganglia and efferent target monoaminergic centers of brainstem (i.e. areas that contain dopaminergic neurons and serotonergic neurons) partly through the RMTg. CPu, caudate and putamen; DBB, diagonal band of Broca; GPb, border region of the globus pallidus; IPN, interpeduncular nucleus; LPO, lateral preoptic area; RMTg, rostromedial tegmental nucleus; SNc, substantia nigra pars compacta. Image borrowed from: Hikosaka J Neurosci 28: 11825-9, 2008.

1.4.4 LHb-VTA circuit

The LHb is a key modulator of midbrain reward circuitry. In contrast to midbrain DA neurons, neurons in the LHb are inhibited by cues that predict reward and excited by the omission of reward (Matsumoto and Hikosaka, 2007). Interestingly, the excitation of LHb neurons during reward omission occurs prior to the inhibition of dopaminergic neurons, which leads to the hypothesis that LHb neurons may modulate VTA dopaminergic neurons. Several theories strengthen the claim that electrical stimulation of LHb inhibits midbrain dopaminergic neurons (Christoph et al., 1986; Ji and Shepard, 2007), whereas pharmacological silencing of LHb induces dopamine release in ventral striatum (Lecourtier et al., 2008). Collectively these studies indicate that LHb encodes negative reward prediction errors and may negatively modulate midbrain dopaminergic neurons in response to aversive stimuli.

Glutamatergic cells mainly populate LHb. However, the fact that activation or inhibition of LHb causes inhibition or excitation, respectively, of DA neurons, has only recently been

unraveled. GABA synapses onto midbrain DA cells (Bayer and Pickel, 1991) are abundant and they arise from both extrinsic and intrinsic sources (Smith and Bolam, 1990; Johnson and North, 1992b; Charara et al., 1996). Those GABA neurons are able to send axon collaterals on VTA DA neurons (Omelchenko *et.al.,*2009) which can form extensive local plexuses (Mailly et al., 2003). Collectively those data suggest that LHb neurons exert influence upon DA neurons through a local disynaptic circuit.

The LHb also sends glutamatergic projections to rostromedial tegmental nucleus (RMTg, located posterior to VTA and is referred to as the tail of the VTA), which comprises of GABAergic neurons (Balcita-Pedicino et al., 2011 ; Stamatakis and Stuber, 2012). In vivo activation of LHb neurons that project to VTA (Lammel et al., 2012), or RMTg – projecting LHb glutamatergic terminals, produces aversive behavior and promotes an adaptive motivated behavior in order for further activation of this circuit to be avoided (Stamatakis and Stuber, 2012). The fact that GABA cells of RMTg inhibit midbrain DA neurons (Matsui and Williams, 2011), renders RMTg as an also possible intermediate structure through which the LHb inhibits midbrain DA neurons.

Afferents from VTA arise to LHb (Gruber et al., 2007; Phillipson and Griffith, 1980; Skagerberg et al., 1984). Those projections comprise the 30%–50% of LHb-projecting dopaminergic neurons (Gruber et al., 2007; Skager- berg et al., 1984).

Electrical stimulation of midbrain DA neurons decreases the firing rate of LHb neurons (Shen et al., 2012). Furthermore, selective optogenetic activation of this projection inhibits LHb neurons and promotes reward-related behavior (Stamatakis *et.al.,* 2013). This action is mediated by GABAA receptors and leads to disinhibition of VTA dopaminergic neurons (Stamatakis *et.al.,* 2013). Overall those data demonstrate that LHb and midbrain interact, with the VTA projections to LHb having a critical role in the classical midbrain reward circuit.

1.4.5 GPi-LHb circuit

Most of the LHb-projecting GPi neurons are excitatory, although it is hypothesized that a minority of GPi neurons form inhibitory connections with LHb as indicated by studies conducted in rats (Shabel et al., 2012) and lampreys (Stephenson-Jones et al., 2013). Neurons from the border region of globus pallidus internal segment (GPi) project to LHb and

most of those projections encode negative reward prediction errors in a similar way with the LHb neurons (Hong and Hikosaka, 2008a). In response to the reward omission, GPi neuronal activation precedes the activation of LHb neurons (Hong and Hikosaka, 2008a).

One candidate source that provides signals to GPi-LHb circuit to create reward prediction errors is the striatum. Physiological studies showed that striatal neurons exhibit sensory responses driven by expected reward values (Kawagoe et al., 1998; Oyama et al., 2010) and they are substantially activated when predicting actions, sensory stimuli or reward (Hikosaka et al., 1989; Lauwereyns et al., 2002; Lau and Glimcher, 2008; Hori et al., 2009). The output neurons in the striatum are the medium spiny neurons, which are GABAergic inhibitory neurons (Tepper and Bolam, 2004), therefore striatal stimulations induce inhibitory responses in the border regions of GPi. The striatum is composed of the patch structures (striosome) and the surrounding areas (matrix) (Graybiel and Ragsdale, 1978; Gerfen, 1984) and it is hypothesized that the effects of the striatum stimulation are mediated by the striosomes. The striosomes receive inputs from limbic areas (Gerfen, 1984; Ragsdale and Graybiel, 1988; Eblen and Graybiel, 1995), suggesting that they carry emotional or motivational signals. In this direction, rats quickly learned to self-stimulate the striosome but not matrix regions (White and Hiroi, 1998). Striatal neurons from striosome project to the rostral part of GPi, which in turn strongly connects to LHb (Graybiel and Ragsdale, 1978; Rajakumar et al. 1993). It is thus expected that the striosome-GPi-LHb circuit contributes to the formation of negative reward prediction error signals in the LHb.

1.4.6 LHb and reward

LHb has recently drawn attention due to its influence on the reward-related dopaminergic cell activity of VTA (Shepard *et.al.,* 2006). LHb projections to the midbrain dopamine neurons are encoding negative outcomes regarding reward-related behaviors. The pivotal study of Matsumoto and Hikosaka using in vivo electrophysiology in primates indicated that neuronal activity in LHb is increased by negative reward prediction errors and decreased by positive reward prediction errors, while the opposite applies for the VTA dopaminergic neurons (Matsumoto and Hikosaka, 2007). In this study rhesus monkeys were trained to respond with saccades to visual cues predicting either rewarding or non-rewarding outcomes (Figure

5). During the non-rewarding trials, the response from the habenular neurons was excitatory and occurred prior to the inhibitory response of midbrain dopaminergic neurons (Figure 6). However, in the rewarded trials, the excitatory response of dopamine neurons, started earlier than the inhibitory response of habenular neurons (Figure 6).

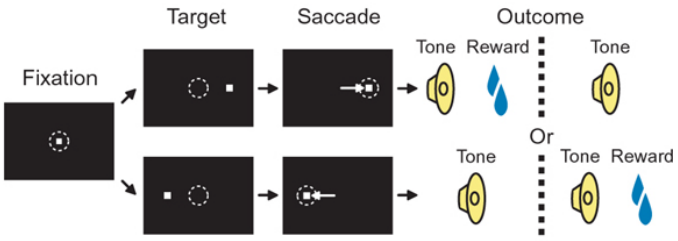

Figure 5. Schematic representation of the 'reward-biased visual saccade task'. Monkeys were trained to quickly make saccades towards an either right or left moving target. The correct saccades followed a tone stimulus, 200ms later. Saccades to one position indicated the delivery of reward, whereas to the other position were not rewarded. Thus, depending on the saccade direction, the target instructed the reward contingency (reward or no-reward). Borrowed from: Matsumoto and Hikosaka, Nature 447: 1111-5, 2007.

These data indicated that activation of LHb has an inhibitory effect upon dopaminergic neurons during the non-rewarded trials, but the inhibition of LHb could not initiate the excitatory response of dopamine neurons during the rewarded trials. Moreover, under the same conditions, habenular and dopaminergic neurons exhibited opposite firing patterns (Figure 6). Therefore, it appears that LHb conducts information to the midbrain reward circuits regarding salient environmental stimuli, which are in fact negative reward-related signals (Matsumoto and Hikosaka, 2007).

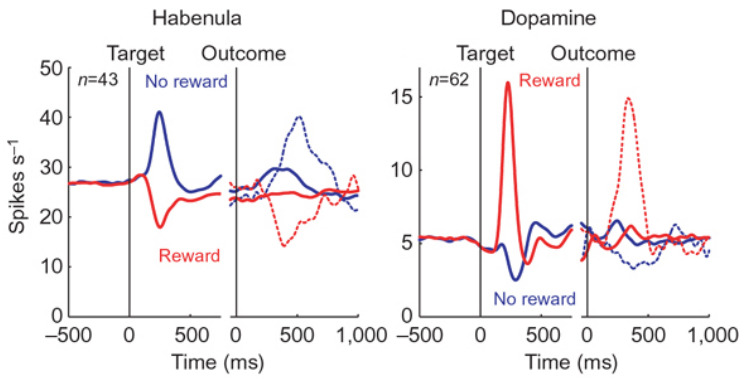

Figure 6. Comparison of lateral habenula neurons and dopamine neurons during reward. The activity of 43 habenular and 62 dopaminergic neurons was recorded in monkeys performing the reward-biased visual saccade task. (Left) Lateral habenula neurons are phasically decreased and increased during the animal's response to the saccade target in rewarded and unrewarded trials respectively. (Right) Dopamine neurons exhibited similar activity patterns compared to lateral habenula neurons, but in an exact opposite manner – they were phasically increased and decreased during rewarded and unrewarded trials respectively. Borrowed from: Matsumoto and Hikosaka, Nature 447: 1111-5, 2007.

1.4.7 LHb in depressive disorders

Studies conducted in monkeys and rodents have linked the normal function of the LHb to the processing of rewarding/punishing information. As aforementioned, the absence of reward and the expectation of punishment are sufficient to strongly activate the LHb.

Furthermore, several lines of evidence support the hypothesis that the hyperactivity of LHb may play an important role in depression. FMRI studies in humans suffering from major depression disorder and also studies regarding metabolic labeling in animal models of depression have shown increased LHb activity in these disorders (Morris JS. *et.al.,* 1999; Shumake J. *et.al.,* 2003). On the other hand, lesion studies in LHb of rodents seem to eliminate depressive-like symptoms (Yang LM. *etal.,* 2008; Anat J. *et.al.,* 2001). In a clinical case study in which the habenula was inactivated by deep brain stimulation (DBS) the result was the full remission of major depression (Sartorius, 2010). Remarkably, inadvertent pause of DBS (which was unknown to the patient) brought back depressive symptoms within a few days. The symptoms were then ameliorated by restarting the DBS.

A number of recent studies in rodents suggest that hyperactivity of LHb neurons may contribute to depression (Figure 7.). One study, in rodent models of depression (Li B. *et.al.,* 2011), revealed enhanced excitatory projections onto LHb neurons that provided inputs to

the VTA. These VTA projecting LHb neurons may selectively excite mPFC-projecting dopaminergic VTA neurons that produce aversion in rodents (Lammel S. *et.al.,* 2012) and could potentially play a role in depression (Mizoguchi K. *et.al.,* 2000). Another study showed that exposure of mice to experimental procedures that are modeling depression, enhanced LHb excitatory synaptic input onto RMTg neurons (Stamatakis *et.al.,* 2012), which would then be expected to eliminate dopaminergic activity of neurons in the VTA (Hong S. *et.al.,* 2011) and may be involved in cognitive impairments observed in depression (Russo *et.al.,* 2013).

Collectively, these studies in primates and rodents examining the normal and abnormal functioning of the LHb, suggest that aberrantly overactive LHb neurons may produce an aversive state of constant disappointment, which may also be a contributor to human major depression disorder.

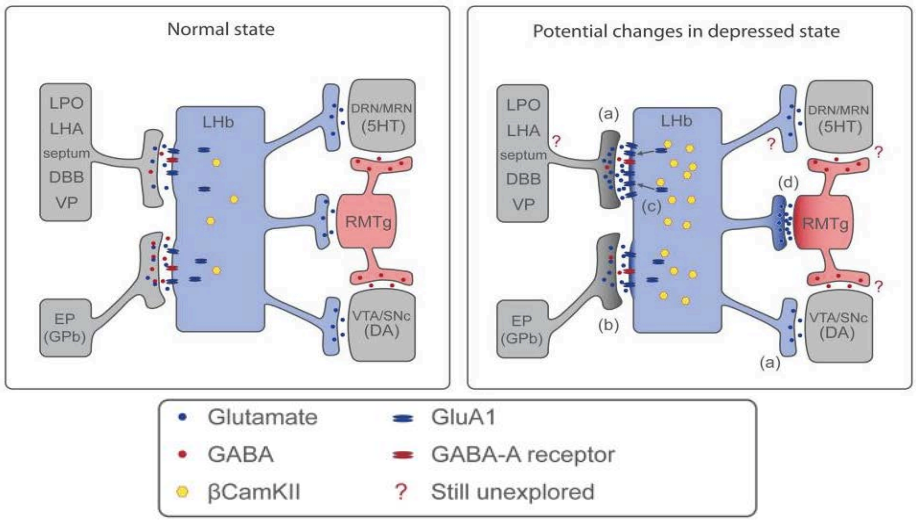

Figure 7. Potential hyperactive synapses in human depression. Schematic showing main inputs and outputs from the lateral habenula (LHb) in normal state and potential cellular and molecular changes in depressed state. Dark shading represents overactive synapses. Depressed state may include increases in the excitatory drive onto LHb and increased LHb output to RMTg and VTA. EP, entopeduncular nucleus; GPb, globus pallidus, border region; LPO, lateral preoptic area; LHA, lateral hypothalamic area; DBB, diagonal band of broca; VP, ventral pallidum; DRN/MRN, dorsal and median raphe nucleus respectively; RMTg, rostromedial tegmental neucleus; VTA, ventral tegmental area; SNc, substantia nigra pars compacta. Image borrowed from: Proulx Nat Neurosci. 17(9): 1146–1152, 2014.

1.5 Methodological background

1.5.1 Optogenetics

The technology of optogenetics is based on the expression of light-activated microbial opsins (Zhang *et.al.*, 2007) channelrhodopsin (ChR2) and halorhodopsin (NpHR) in neurons using genetic approaches. The optogenetic approach allows for the bidirectional modulation of neuronal activity in defined neuronal populations during active animal behavior. ChR2 is activated in the 470nm blue light range and causes neuronal firing with millisecond kinetics and over a wide range of frequencies. NpHR is activated by 580nm yellow light range and leads to neuronal hyperpolarization and inhibition of action potential firing (Figure 8.).

The optogenetic approach described here is a viral based expression of ChR2 (but not NpHR) in specific neuronal populations based on an inducible Cre recombination step in adeno-associated viruses (AAV) (Cardin, Carlen & Meletis *et.al.,* 2009). The methodology comprises of a combination of transgenic mouse lines and Cre-dependent AAV viruses that enable the expression of genes in discrete neuronal populations. This approach allows for the first time the direct investigation of the function of genetically defined neuronal classes. The experimental approach described herein has been successfully implemented in Konstantinos Meletis' and Marie Carlen's laboratories (Karolinska Institutet). The combination of optogenetics and molecular manipulations together with in vivo recordings in mice that engage in relevant behaviors is a very powerful approach to identify the contribution of distinct neuronal populations in specific parts of a task and thereby assign function to neuronal classes during normal behavior. This information can then be used to evaluate the function of the same neuronal classes in animal models of human neuropsychiatric disorders and test the possibility of treating abnormal behavior with optogenetics or pharmacology.

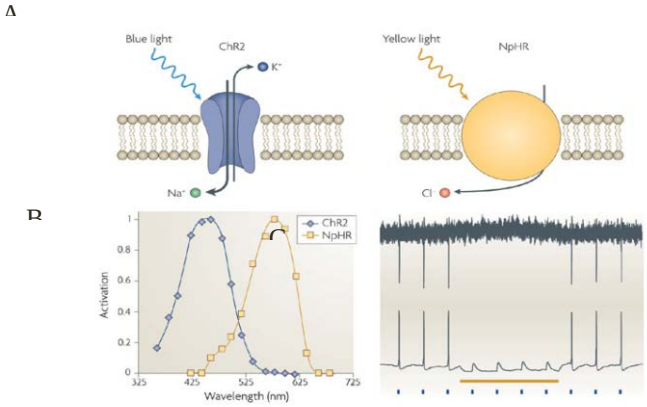

Figure 8. A Schematic of channelrhodopsin-2 (ChR2) and the halorhodopsin (NpHR) pump. Following illumination with blue light (activation maximum 470 nm), ChR2 allows the entry of cations (mostly Na^+ and very low levels of Ca^{2+}) into the cell. NpHR is activated by yellow light illumination (activation maximum 580 nm) and allows the entry of Cl^- anions. B Action spectra for ChR2 and NpHR. The excitation maxima for ChR2 and NpHR are separated by 100 nm, making it possible to activate each opsin independently with light. C Cell-attached (top) and whole-cell current-clamp (bottom) traces from hippocampal neurons showing all-optical neural activation and inhibition. Blue pulses represent the blue light flashes used to drive ChR2-mediated activation and the yellow bar denotes NpHR-mediated inactivation. Image borrowed from: Zhang *et.al.*,Nat Rev Neurosci 8: 577-81, 2007

1.5.2 Anatomy- Rabies tracing system

Circuit connectivity is traditionally described anatomically with chemical tracers either retrograde or anterograde which provide a visualization of axons and their targets. Konstantinos Meletis' and Marie Carlen's laboratories (Karolinska Institutet) have established a genetically based method for the anatomical dissection of circuits using genetically modified rabies viruses that display specific transsynaptic retrograde spread in transgenic mice (Wickersham *et.al.*, 2007; Wickersham *et.al.*, 2010). In this method, rabies virus has been genetically modified to express fluorescent markers (e.g. mCherry, EGFP) and light-activated opsins (e.g. ChR2, NpHR3.1) giving the possibility to both map the anatomical and functional connectivity of circuits and to optogenetically manipulate the traced connections. The experimental paradigm is based on the Cre-dependent expression of a) the avian receptor (TVA) required for internalization of the rabies virus as well as b) the glycoprotein G (GP) required for transsynaptic, monosynaptic spread of the virus. Thus the expression of the receptor TVA and the glycoprotein G is restricted to Cre-expressing neurons (e.g. glutamatergic or GABAergic neurons) from the Cre-dependent AAV virus (AAV

DIO TVA-GP). The AAV DIO TVA-GP virus ensures a strictly monosynaptic retrograde transport of the rabies virus to the upstream population (Figure 9.).

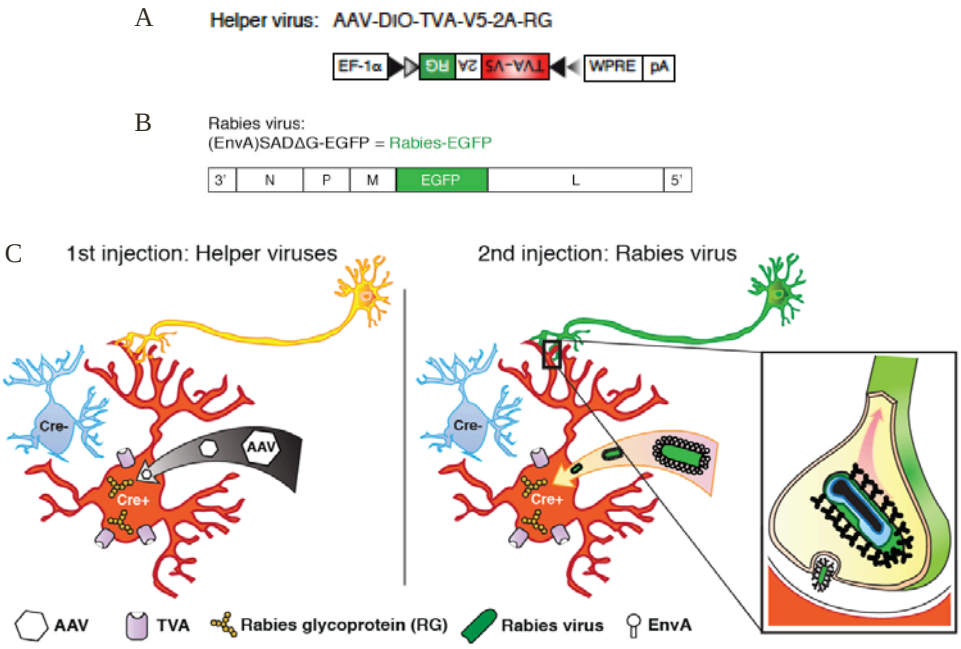

Figure 9. Experimental strategy for the monosynaptic tracing of the Rabies virus system. (A) AAV helper viruses with Cre-dependent expression of TVA receptor and RG. (B) Genetically modified rabies virus is pseudotyped with EnvA. The RG gene is replaced by EGFP. (C) The first injection (AAV helper viruses) induces selective expression of TVA and RG in Cre-expressing neurons (Cre+). The second injection (EnvA-coated rabies virus) results in rabies uptake by TVA-expressing neurons and transsynaptic retrograde transport of RG-coated rabies virus into upstream input neurons. Image borrowed from: Pollak Dorocic *et.al.,* Neuron 83:663-78, *2014*

1.5.3 Behavioral tasks

Many human pathologies have been addressed in mice by means of different behavioral paradigms. Many of these tests exploit the natural behavior of mice for example in exploratory activity, social investigation or their aversion towards open, brightly lit or elevated spaces. The fact that basic physiological mechanisms controlling fear, reward or other behaviors in mice can be equated to similar mechanisms in humans constitute these paradigms valid for use. Specifically, in the current project, we used the Condition Place Preference test (CPP) in order to examine factors like aversion and preference. CPP is the simplest and most suitable test applied to measure preference or avoidance behavior. In CPP a specific neuronal circuit is excited or inhibited in one of the compartments of the behavioral apparatus and the subject is tested for its preference or avoidance of that

compartment. More details on this behavioral paradigm are presented in Chapter 2 (Materials and Methods).

1.6 Aim of the study

As aforementioned, LHb is a key modulator of midbrain reward circuitry and its disregulation has been associated with mood and psychiatric disorders. Recent studies pinpoint GPi as a key source for reward signals to LHb. The goal of this study is to identify and study the different neuronal populations of the GPi that control LHb. Our approach involves the anatomical and the functional-optogenetic dissection and manipulation of the circuits regulating reward. In particular with the use of anterograde viral based tracers we are aiming to define the populations in GPi and characterize their projections to LHb. Furthermore we employ the transsynaptic rabies tracing system that allows us to directly identify all the synaptically connected neuronal populations that control the activity of the LHb-projecting GPi neurons. Finally, in addition to the anatomical characterization, we aim to functionally dissect the role of each presynaptic population for reward related behaviors using optogenetics.

With this project we aim for the first time to probe the molecular identity of neuronal subtypes involved in reward and define their contribution during reward related behavior.

Chapter 2. Materials and Methods

2.1 Subjects

Adult mice (25-32 gr) were group housed (3–5 mice/cage) using Makrolon type III cages, under standardized conditions until any experimental procedure and maintained on a reverse 12-hr light cycle (lights off at 8:00) with *ad libitum* access to food and water. Animal experiments were carried out following guidelines of the Stockholm municipal committee.

VGluT2-Cre+/- (Hippenmeyer *et.al.,* 2005) mice expressing Cre recombinase specifically in VGluT2 neurons were obtained from Jackson Laboratories. Heterozygous mice were crossed to wild type C57BL/6N mice to maintain the line and obtain heterozygous mice that were used in the study.

2.2 Genotyping of transgenic animals

DNA samples were prepared from mouse ear snips. The tissues were placed in 150µl of Alkaline lysis reagent (25mM NaOH, 0.2mM Na2-EDTA 2H2O, in extra pure water, pH:12) and heated at 95°C for one hour. After heating, samples were vortexed vigorously for 10sec and were neutralized with 150µl of Neutralization reagent (40mM Tris-HCl, pH:5). DNA samples were analyzed by PCR, which was performed using Quick-Load Taq 2× Master Mix and primers specific for Cre allele and internal control (Carlen *et.al.,* 2012). PCR conditions: denaturation at 94°C for 5 min, 94°C for 30 s, 58°C for 30 s, 68°C for 4 s followed by 30× 94°C for 30 s, 68°C for 5 min. PCR products were analyzed by electrophoresis on 2% agarose with GelRed (Biotium) 1:10.000. Two bands were generated in Cre positive animals, with the lower band referring to the Cre product (500bp) and the upper band to the internal control.

2.3 Stereotactic injections and Fiber implantation

VGluT2-Cre mice (Zhuang et al., 2005) 2 to 7 months old were used for monosynaptic tracing

and behavior. Mice were anesthetized with isoflurane and placed in a stereotaxic frame. For the tracing study, a volume of 0.2µl of AAV-DIO-TVA-V5-t2A-RabiesG virus was injected in GPi and twenty-one days later, 0.2µl of EnvA-coated Rabies-EGFP virus was injected with a 10° angle in LHb. For the optogenetic assessment of behavior, 0.4µl of ChR2-mCherry virus was injected in GPi while optic fibers were implanted in LHb twenty-five days later. The coordinates used were: for GPi (in mm from bregma): -1,. anterior/posterior, ±1.75 medial/lateral, 4.1 dorsal/lateral. For LHb monosynaptic tracing (in mm from bregma): -1.65 anterior/posterior, ±0.92 medial/lateral, 2.4 – 2.8 dorsal/lateral, injections with a 10° angle. For LHb fiber implantation (in mm from bregma): -1.65 anterior/posterior, ±0.92 medial/lateral, 2.44 dorsal/lateral, fibers implanted with a 10° angle.

For the anterograde tracing experiments he 0.7 µl of AAV-flexi-tdTomato virus were injected in Gpi of VGluT2-Cre and VGAT-Cre according to the previously mentioned coordinates.

2.4 Histology, Immunohistochemistry and microscopy

Mice were deeply anaesthetized with pentobarbital and then transcardially perfused with PBS followed by a fixative solution (a mixture of 4% (w/v) Paraformaldehyde, 15% (v/v) Picric acid, 0.05% (v/v) Glutaraldehyde in PBS). Brains were removed and remained overnight in fixative solution for post-fixation. 40µm-thick coronal sections throughout GPi and LHb, were cut using a vibratome and collected in PBS. Sections were incubated on a shaker, for one hour in 0.3% TritonX-100 in Tris –buffered saline (38mM Tris-HCl, 8mM Trizma base, 120mM NaCl in extra pure water) and treated with a prewarmed (40^{o}C) antigen retrieval solution (10mM sodium citrate, 0,05% Tween20, pH:6) for 1-2 minutes. In order to block the non-specific antibody binding, sections were incubated in 5% Normal Donkey Serum in TBST (0,3% TritonX-100 in Tris –buffered saline), for one hour at room temperature. Sections were subsequently incubated overnight with primary antibodies: goat anti-SOM IgG 1:500 and guinea-pig anti-PV IgG 1:1.500 in TBST. The next day they were washed twice for 10 minutes in TBST and incubated on a shaker at room temperature for 5 hours with the secondary antibodies: anti-goat Cy5 1:500 and anti-guinea-pig 488 1:500. The

sections were then washed sequentially in TBST, TBS, PBS, for 10 minutes in each solution. For the mounting of the sections, the Slow Fade antifade mounting solution was used. For all the immunohistochemical procedures conducted in this study, the same protocol was used regardless the various combinations of antibodies used. For the c-Fos staining, the primary antibody used was the rabbit anti-c-Fos (1:1000) in combination with the secondary antibody Alexa 488 anti-rabbit (1:500) and prior to the mounting step, slices were treated with the fluorescent stain Dapi in order to visualize cell nuclei (Dapi dilution 1:50.000 in PBS). Z-stack and tiled images were captured on a Zeiss LSM 5 Pascal confocal laser-scanning microscope using a 20x, objective. Images were acquired using identical pinhole, gain, and laser settings for all brain regions and analyzed using ImageJ software. Segmentation of cell bodies and registration of position on the Allen mouse reference atlas was performed using custom software.

2.5 Behavioral experiments

Behavioral tests were conducted between 12:00 and 16:00 p.m. The mice were transferred to the testing room at least 1 h prior to the test in order to habituate to the surroundings. For the Real Time Place Preference (PP), a rectangular black plexiglas apparatus (48 x 18 x 23 cm) was used. The device consisted of two chambers, connected with a constantly open door so that the subject was allowed free access to either of the compartments. The protocol was conducted in three days. The first day animals underwent the PP test while connected with the optic fibers, yet without being stimulated by light, in order to ensure that subjects did not exhibit preference for either of the two chambers. The second day, the animals were stimulated with light (447nm, 60Hz, 1ms pulse) only when entering and remaining in the right compartment, while no stimulations occurred in the left compartment. The third day, the procedure was repeated but the stimulation was on, in the left compartment and off in the right one. Time spent and distance covered, in each compartment, were measured throughout a 20 - minutes period using an automated video tracking software (Biobserve, Germany).

Chapter 3. Results

3.1 GPi neuronal populations

In order to define the identity of neurons residing in GPi, in terms of expression of specific markers and of their topographic distribution throughout the GPi, a Cre-dependent anterograde tracer (Flex-TdTomato) was used in combination with immunohistochemistry. In order to define the distribution of these cell populations and to examine the presence of axonal terminals within the LHb, we stained brain slices containing GPi and LHb, from VGluT2-cre and VGaT-cre animals injected with AAV-Td-Tomato virus in Gpi and counterstained them with antibodies against parvalbumin. Our data indicate that GPi neuronal populations comprise of VGluT2^{+} glutamatergic cells, parvalvumin (PV^{+}) gabaergic cells and also PV^{-} gabaergic cells (Figure 10, B). From this experiment we were able to show that glutamatergic cells start appearing in the most anterior and dorsolateral part of GPi and their numbers are higher in the central parts of Gpi and lower posteriorly. On the other hand PV^{+} cells appear in more posterior parts of GPi with higher numbers occuring in the posterior ventromedial part of GPi (Figure 10, A). The glutamatergic neurons outweigh in numbers the PV neurons in the most anterior part of GPi and this is reversed more caudally. Stainings for PV in VGluT2-Cre mice showed that glutamatergic neurons of GPi (Td-Tomato positive cells) do not express PV and vise versa (Figure 10, B and C). Furthermore, stainings for PV, that were conducted in VGaT-Cre mice revealed an almost absolute colocalization of PV positive cells with the Td-Tomato positive cells, thus the vast majority of PV cells in GPi are GABAergic (Figure 10, B). Finally, both VGluT2 positive and VGaT positive fibers, were found to arborize within the LHb (Figure 10, D).

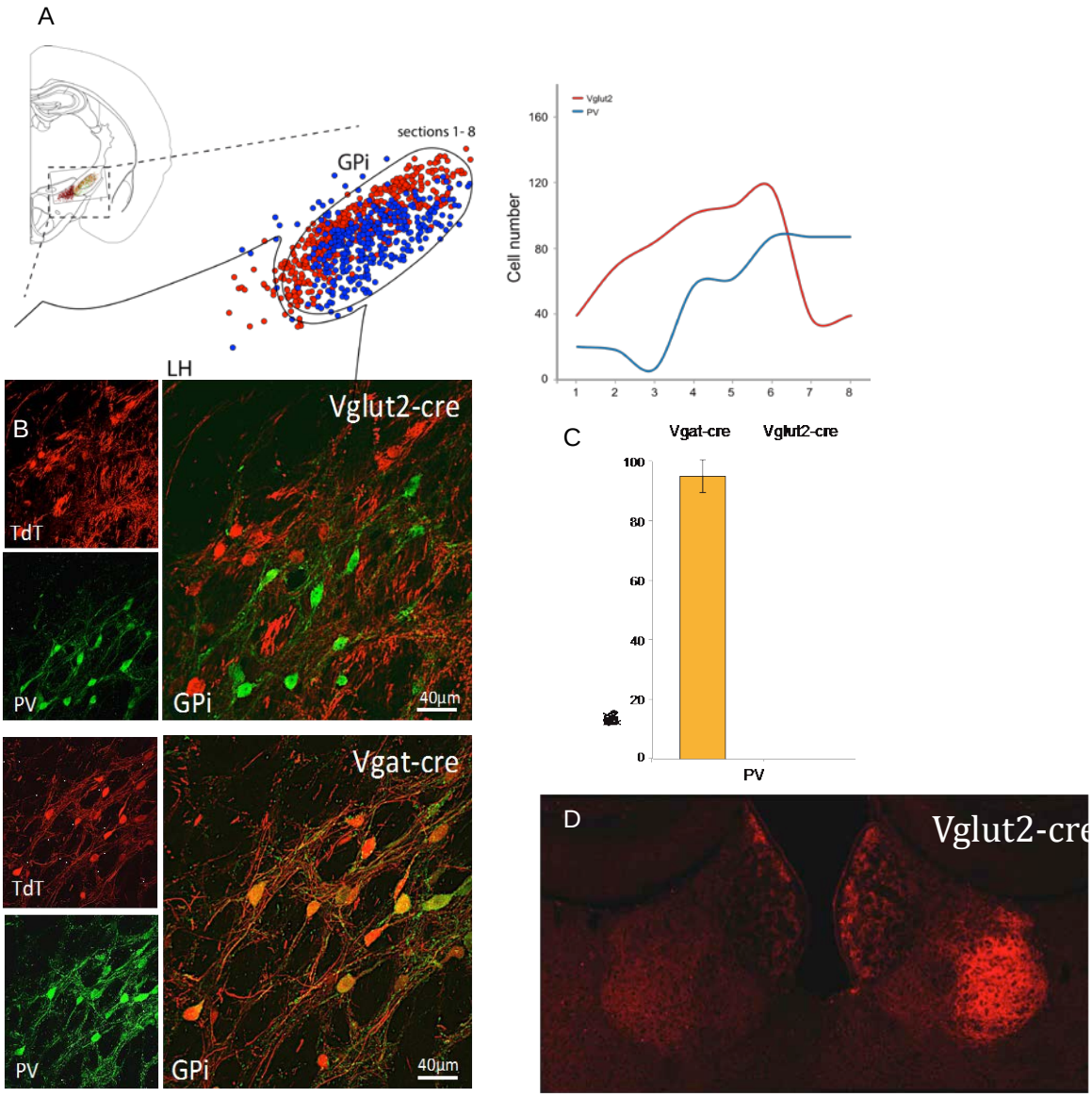

Figure 3. Topographic characterization of VGluT2, VGaT and PV populations in GPi. A. Schematic representation of VGluT2 and PV cell populations in GPi (left), throughout an anterior – posterior axis (right). B. Confocal images of Td-Tomato injections in the GPi of VGluT2-cre and VGaT-cre animals respectively. Colocalization with Alexa488 (PV). C. Histogram representing the percentage of colocalization of PV cells with VGaT and VGluT2 positive cells respectively. D Figure showing the glutamatergic inputs from GPi to LHb.

3.2 GPi neurons that project to LHb receive direct input from GPe and striatum

Cell type specific retrograde tracing was performed using our two-vector system. AAV-DIO-TVA-V5-2A-RG vector was injected in GPi and three weeks later Rabies-SADΔG-EGFP was injected in LHb of Vglut2-cre mice to target the glutamatergic population of GPi. VGluT2+

fibers were found in the most lateral part of LHb (Figure 11, C). Projections from GPi to the MHb were not observed. The neurons arising from GPi, were characterized by a dense EGFP expression throughout the neuronal somata and axons (Figure 11, D).

The efficiency of the two-vector system was assessed by checking the colocalization of EGFP (expressed by the rabies virus) with the V5 protein tag (expressed by the AAV virus), on brain slices containing GPi (Figure 11, E, F and G). The coexpression of the two markers revealed the population of GPi neurons that directly projects to LHb (starter population). More than 80% of EGFP positive neurons within the GPi were also positive for V5.

The monosynaptic spread of the Rabies virus revealed that LHb projecting GPi neurons, receive direct input from the GPe and the CPu of the striatum (Figure 11, H and I). The neuronal profile of the GPe and CPu projections to GPi was identified with immunochistochemistry. CPu is compartmentalized into islands of patches and the surrounding matrix area. In the patch compartment of striatum, the Mu opioid receptor is highly expressed (Herkenham and Pert, 1981). Stainings for Mu opioid receptor showed that a proportion of the EGFP positive neurons of CPu were colocalized with the Mu receptor, while the rest were situated in the surrounding areas. Thus, the striatal inputs to LHb projecting GPi neurons, are arising from both the patch and matrix compartments (Figure 11, J).

In the same direction, markers for the main neuronal populations of GPe were used in order to characterize the profile of the GPe to GPi projecting neurons. GPe is populated by PV positive, PV negative and Foxp2 positive neurons (Dodson *et.al.,* 2015). Stainings for PV and Foxp2 showed no co-expression of EGFP cells with FoxP2 whereas EGFP cells were either positive or negative for PV (Figure 11, K). Therefore, the GPe inputs to LHb projecting GPi neurons, involve PV positive and non-PV cells while the Foxp2 positive neuronal population is not included in this circuit.

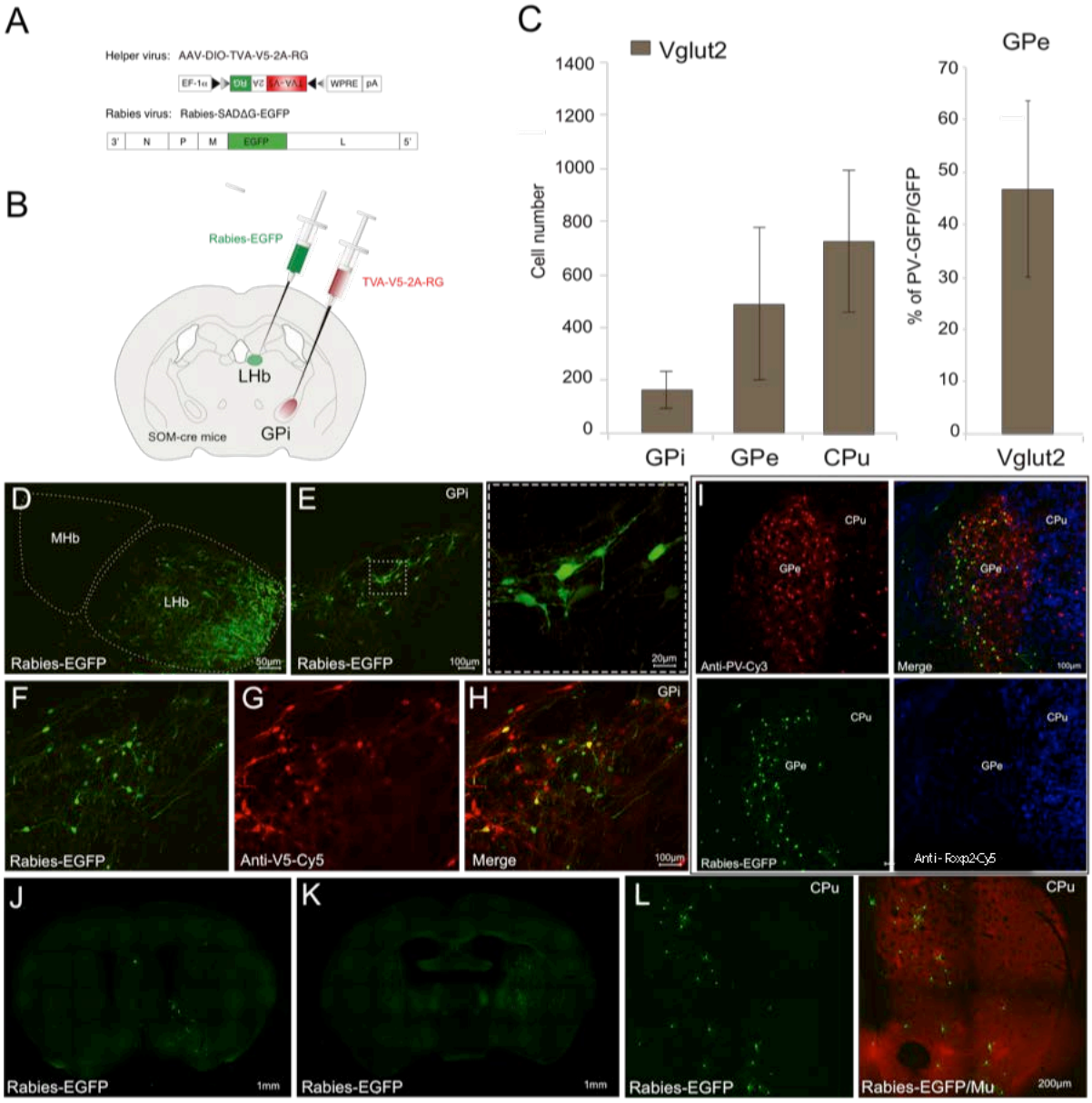

Figure 4. Tracing of the GPi inputs to LHb. A&B. Schematic representation of the injection sites: TVA-V5-2A-RG virus in GPi, Rabies-EGFP virus in LHb. C Left: Total numbers of glutamatergic cells that arise in GPi and project to LHb, or arise in GPe/CPu and project to the LHb projecting GPi neurons. Right: The percentage of glutamatergic cells in GPe that also express PV. D&E The main recipient of GPi inputs is the lateral part of LHb (less dense inputs to the medial part of LHb) but not the MHb. F,G&H Starter population of GPi neurons that project to LHb. Colocalization of Rabies-EGFP with the V5 epitope which is expressed by cells infected with the helper AAV virus. I GPe inputs to GPi-LHb circuit. Cells populating the GPe are PV positive, while there are also PV negative and Foxp2 positive cell populations. GPe neurons projecting to GPi-LHb circuit are PV positive as well as PV negative, but Foxp2 negative. J&K The GPi neurons that project to LHb receive input from GPe (J) and CPu (K) respectively. L CPu inputs to GPi-LHb circuit are provided by the striatal matrix compartment, as well as by projection neurons residing in the striatal patch compartment (striosomes), as indicated by the colocalization of Rabies-EYFP with the Mu opioid receptor antibody.

3.3 Activation of glutamatergic GPi neurons projecting to LHb causes aversion

In order to dissect the function of the circuit GPi-LHb, ChR2-EGFP was injected bilaterally in the GPi of VGluT2-Cre mice and optic fibers were implanted in LHb (Figure 12, A,B,C and D). A week post fiber implantation the mice were tested in real time place preference and elevated plus maze . Light activation of GPi glutamatergic neurons projecting to LHb by blue laser (475nm) induced a strong aversive response for the stimulation paired compartment (Figure 12, E and F). Mice avoided the compartment where laser stimulation was evoked and spent more time in the one with no stimulation (Figure 12, G). This aversive response positively correlated with the stimulation frequency, starting with a moderate aversion effect at 2.5Hz and reaching complete avoidance of the stimulation compartment at 60Hz (Figure 12, H).

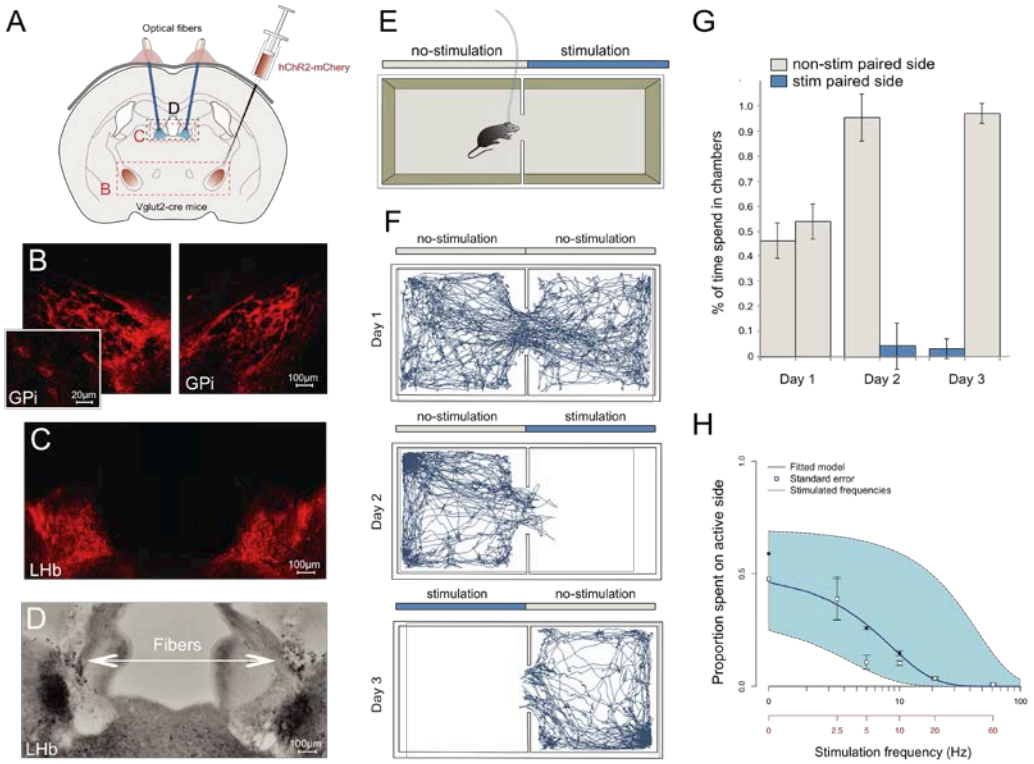

Figure 5. Induction of strong avoidance response produced from light activation of Vglut2+ GPi afferents in LHb. A, Schematic showing the injection and implantation sites of AAV-DIO-hChR2-mCherry and optical fibers in GPi and LHb, respectively. B-D, Confocal images showing bilateral injection sites of AAV-DIO-hChR2-mCherry virus in the GPi (B, insert shows higher magnification of GPi labeled cells), GPi afferents in LHb (C) and the

traces of the 200µm core optical fibers in LHb (D). E, Schematic showing the place preference behavioral box. F, Diagrams show the track of an implanted mouse in place preference behavioral box. Left to right: day 1 no light (open bars), day 2 light activation in the right compartment (blue bar), day 3 light activation in the left compartment (blue bar). G, Diagram shows average responses (n=6, Error bars represent SD). H, Optical stimulation frequency analysis of behavioral responses (n=2)

Similar results were obtained from the elevated plus maze. In this behavioral paradigm mice were stimulated by light while entering the closed arms of the elevated plus maze, but not when they stayed in the open arms. We hereby tried to test whether the mice maintained the aversive phenotype regarding laser stimulation and "overcome" their innate aversion for elevated and exposed areas by spending more time in the open arms of the devise. Indeed, mice seemed to prefer being exposed in the center and open arms of the devise rather than spend time in the stimulation-coupled closed arms (Figure 13).

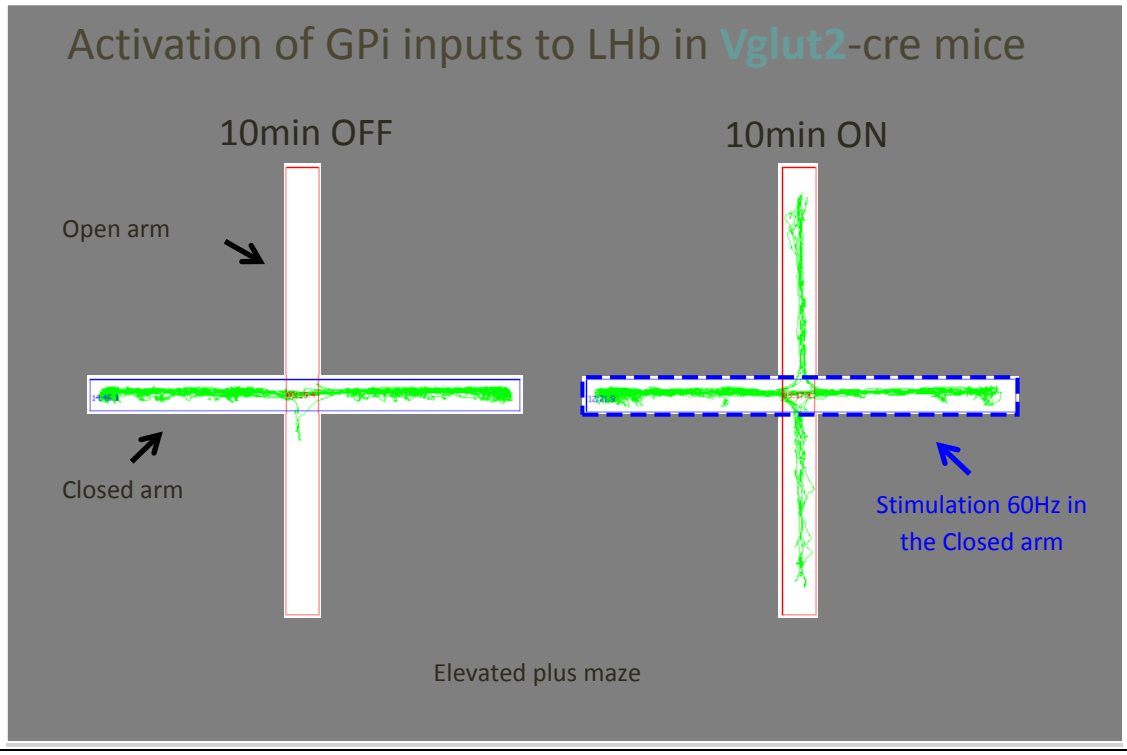

Figure 6. Activation of GPi inputs to LHb in VGluT2-cre mice. Mice spent more time in the open arms when the lazer was ON. Blue lazer stimulation (447nm), 60Hz.

Chapter 4. Discussion

For the subcellular characterization of neurons residing in GPi and arborizing in LHb, a combination of viral anterograde tracing along with immunohistochemistry techniques was used. The AAV-Flex-Td-Tomato virus was injected in GPi of VGluT2-Cre and VGaT-Cre mice and allowed us to specifically label either the glutamatergic or the GABAergic populations of GPi respectively, thus enabling also to visualize GPi projections to LHb. Indeed, both VGluT2^{+}-Td-Tomato and VGaT^{+}-Td-Tomato fibers were found to send synaptic inputs to LHb. GPi was thought to be a putative gabaergic nucleus involved in motor related commands as one of the main outputs of basal ganglia. Only recently literature was enriched by Shabel and co-workers who were the first to optogenetically show that there is also a glutamatergic population within GPi (Shabel *et.al.,* 2012). More specifically they injected a cholera toxin retrograde tracer in LHb and shown that 70% of LHb projecting GPi cells were VGluT2^{+}. Shabel's work proposed that this glutamatergic population of GPi provides inputs to LHb, the activation of which promotes aversive behaviors. In this project we directly targeted the glutamatergic popolulation by using VGluT2-Cre mice and a Cre dependent ChR2 virus and we showed that optogenetic stimulation of LHb resulted in a strong aversive behavior in accordance to Shabel's hypothesis. The difference in the strength of the behavioral response might be due to the fact that we targeted specifically VGluT2+ cells whereas Shabel et al. targeted a mixed population by using a general promoter for the ChR2 virus.

Furthermore we characterized the topographic orientation of neuronal subpopulations within the GPi. We utilized an anterograde tracer in VGluT2-Cre mice along with immunohistochemistry for PV. Our findings revealed that VGluT2^{+}-Td-Tomato cells occupy the most anterior and dorsolateral part of GPi, while PV^{+} neurons appear in the most posterior and ventromedial territories of the nucleus. Additionally, VGluT2^{+}-Td-Tomato neurons lay dorsally of the PV^{+} population and there is no colocalization between them. Instead, approximately the 90% of PV^{+} neurons where also VGaT^{+}-Td-Tomato, indicating that almost all PV^{+} cells are GABAergic. Only a small proportion of the VGaT^{+}-Td-Tomato GABAergic neurons of GPi projects to LHb consistent with previous studies (Araki *et.al.,* 1984). The motor-related part of GPi is mainly comprised of PV^{+} neurons. Assuming that the major PV^{+} population of GPi is related primarily with the motor functions of the nucleus, our

findings suggest that glutamatergic and PV^+/GABAergic populations are topographically separated and this is preferably due to the differential information they carry, with a minor GABAergic GPi input to LHb probed to be involved in limbic functions.

The GPi is known to send reward related signals to LHb (Hong S. *et.al.,* 2008) through glutamatergic input, the stimulation of which produces a strong aversion phenotype (Shabel *et.al.,* 2012). According to those references, we proceeded by subjecting VGluT2-cre animals to the place preference paradigm, where we specifically stimulated the excitatory inputs from GPi to LHb. Light stimulation with the optical fibers was induced in the LHb in one of the place preference cage compartments and the resulting behavior was always aversion, suggesting that glutamatergic inputs from the GPi to the LHb drive LHb neuronal responses to aversive events. Inspection of the insertion site of the optic fiber showed that they were correctly implanted in the LHb, where the axonal terminals of the AAV infected VGluT2 cells expressing ChR from GPi are present.

Studies in primates revealed that antidromically identified LHb-projecting GPi neurons are strongly modulated by expected reward outcomes (excited by the prediction of no reward and inhibited by the prediction of reward) and their reward-dependent modulations resemble and precede those in LHb neurons (Hong S. *et.al.,* 2008). These findings suggest that GPi may initiate reward-related signals through its effects on the LHb, which then influences the dopaminergic and serotonergic systems.

In order to investigate the monosynaptic inputs to LHb projecting GPi neurons, we performed retrograde tracing using our uniquely developed dual-viral system. That was achieved by injecting the helper virus (AAV-DIO-TVA-V5-2A-RG) in the GPi of VGluT2-cre mice, and the Rabies-EGFP virus in the LHb. Only the LHb projecting VGluT2 cells expressing the TVA receptor would allow the internalization of Rabies virus. Furthermore, expression of the V5 epitope enabled us to identify and separate the primary infected GPi population from the transynaptically labeled input neurons. The results of the Rabies retrograde tracing are consistent with our findings using the anterograde tracer, regarding the fact that the glutamatergic GPi input targets the more lateral part of LHb. The monosynaptic retrograde spread of the Rabies, revealed the direct inputs to LHb projecting GPi neurons. The results indicated that the inputs to LHb projecting GPi neurons are originating from the GPe and the

CPu. Immunostaining with PENK, a marker for the indirect D2 pathway showed that in CPu, EGFP positive cells were both PENK+ and PENK- (unpublished data from Meletis' lab). This shows that LHb projecting GPi neurons receive information from both D1 and D2 striatal medium spiny neurons (MSN) involved in the direct and indirect pathways respectively.

The striatum is composed of the patch structures (striosome) and the surrounding areas (matrix) (Graybiel and Ragsdale, 1978; Gerfen, 1984). The striosomes receive inputs from limbic areas (Gerfen, 1984; Ragsdale and Graybiel, 1988; Eblen and Graybiel, 1995), suggesting that they carry emotional or motivational signals. In order to verify the origin of the GPi projecting striatal neurons, stainings for the patches marker Mu showed that both matrix and striosomes provide information to LHb projecting GPi neurons.

The GPe population is comprised of two major GABAergic cell types: the prototypic and arkypallidal neurons, which exhibit distinct firing patterns (Mallet et al., 2012). Importantly, prototypic and arkypallidal neurons also project to distinct sets of basal ganglia targets; the former cell type innervates the subthalamic nucleus (STN) and basal ganglia output nuclei, whereas the latter only innervates the striatum (Mallet et al., 2012). Arkypallidal and prototypic neurons are defined by their mutually exclusive expression of FoxP2 and Nkx2-1, respectively, with the Nkx2-1 prototypic neurons being further subdivided in Nkx2-1/PV+ and Nkx2-1/PV^- (Dodson et.al., 2015). Our findings indicated that a large cell population distributed in the borders of GPe targets the LHb projecting GPi neurons. Within this population, neurons were found to be PV^+ (~45%) as well as PV^-/$Foxp2^-$. Therefore GPe cells targeting LHb projecting GPi neurons are prototypic and either PV^+ or PV^-. Prototypic GPe neurons exhibit relatively high firing rates when the animal is at rest and, thus, potentially have a wide dynamic range (large negative and positive activity modulations) for encoding behavior. These properties are shared by LHb projecting GPi neurons (Hong S. *at.al,* 2008) and together with our data, showing GPe to GPi projections further promote the notion that GPe activity drives LHb projecting GPi neurons. In contrast, arkypallidal neurons fire at relatively low rates during rest, indicating they have less scope for negative activity modulations (Dodson et.al., 2015). In agreement with this we didn't find any arkypallidal FoxP2+ neurons connecting to LHb projecting GPi neurons. Moreover, prototypic GPe neurons innervate the subthalamic nucleus (STN) as well as output basal ganglia nuclei (GPi and SNC) and, occasionally the striatum (Mallet et al., 2012). A decrease in their firing rate

during movement would thus fit well with the proposed role of the GPe in the classical ‘‘indirect pathway,’’ i.e., disinhibition of STN and output nuclei (Sano et al., 2013), which should ultimately inhibit unwanted actions or terminate action sequences (Gerfen and Surmeier, 2011). Our data suggests that GPe can also carry reward related signals.

Chapter 5. Conclusions

Our results pinpoint the glutamatergic population of Gpi as a key conductor of anti reward signals to the LHb. Furthermore with our unique virus system we reveal for the first time the source of reward signals to reside in the GPe and CPu. Collectively our work promotes the characterization of the neuronal networks involved in reward related behaviors.

5.1 Future goals

The involvement of the GABAergic GPi population that projects to LHb needs to be further characterized, in regards to the specific inputs it receivess and potential involvement in reward-related behaviors. In the same manner, we need to verify the contributions of the main input providers to this circuit i.e. the GPe and the CPu regarding the emotional information they promote. This could be probed by expressing a cre dependent Rabies-ChR2 virus into the specific neuronal populations of GPe and CPu that synapse with the LHb projecting GPi neurons. Thus, by optogenetically activate or inactivate the GPe-GPi and CPu-GPi pathways, we will have functionaly dissected the former circuits. As LHb is implicated in depressive-like behaviors

Chapter 6. References

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