Mapping the Fasciculus Retroflexus

A review on the implications of the lateral habenula in the dopaminergic and serotonergic midbrain areas

MA Thesis
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Figure title page: Staining of the fasciculus retroflexus, results section Kantor *et al.*, Neuron 2004, p. 969

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List of abbreviations

5-CSRTT:	5-choise serial reaction time task
Brn3a:	Product of *Pou4f1* gene/transcription factor
CSPGs:	Chondroitin sulfate proteoglycans
DCC:	Deleted in colorectal cancer
DDC:	Dorsal diencephalic conduction system
D(n):	Neuromere n
DRN:	Dorsal raphe nucleus
E(n):	Embryonic day n
EPN:	entopeduncular nucleus
fMRI:	Functional magnetic resonance imaging
FR:	Fasciculus retroflexus
GABA:	*gamma*-aminobutyric acid
Hb:	habenular nuclei
HRP:	Horseradish peroxidase
HSPGs:	Heparan sulfate proteoglycans
IFN:	Intrafascicular nucleus
IPN:	Interpeduncular nucleus
LHb:	Lateral habenula
LHbL:	Lateral division lateral habenula
LHbLB:	Basal part lateral division lateral habenula
LHbLMc:	Magnocellular part lateral division lateral habenula
LHbLMg:	Marginal part lateral division lateral habenula
LHbLO:	Oval part lateral division lateral habenula
LHbLPc:	Parvocellular part lateral division lateral habenula
LHbM:	Medial division lateral habenula
LHbMA:	Anterior part medial division lateral habenula
LHbMC:	Central part medial division lateral habenula
LHbMMg:	Marginal part medial division lateral habenula
LHbMPc:	Parvocellular part medial division lateral habenula
LHbMS:	Superior part medial division lateral habenula
LR:	Left-right
LTD:	Long-term depression
mdDA:	mesodiencephalic dopaminergic
MFB:	Medial forebrain bundle
MHb:	Medial habenula
MRN:	Medial raphe nucleus
NAcc:	Nucleus accumbens
NMDA:	*N*-Methyl-D-aspartic acid
Nrp1a:	Neuropilin1a
P(n):	Prosomere n
PPI:	Prepulse inhibition
Sema3F:	Semaphorin class 3 F
Sema5A:	Semaphorin class 5 A
SM:	Stria medullaris
SN:	Substantia nigra
SNc:	Substantia nigra pars compacta
TH:	Thalamic nucleus
tVTA/RMTg:	Ventromedial mesopontine tegmentum
VTA:	Ventral tegmental area
VTApn:	Medial paranigral ventral tegmental area

Abstract

There is growing awareness that motivation and reward are important determining factors of our behavior. The habenular complex is uniquely positioned both anatomically and functionally to participate in the circuit mediating some forms of these behaviors. The habenula, a very intriguing and complex structure, is a node linking the limbic and striatal forebrain with midbrain and hindbrain structures. It is composed of bilaterally medial and lateral subdivisions. Because of this complexity in anatomy and connections, the habenula is implicated in a various range of behaviors. Recent studies suggest that the lateral habenula also plays a pivotal role in cognitive behaviors, such as learning, memory, and attention, by influencing the dopamine and serotonin neurons of the ventral midbrain. Besides, via its inhibitory control over these monoaminergic systems, the lateral habenula exerts influence on reward-related behaviors. Dysfunctions of these cognitive behaviors, as well as the motivation and the reward system, are thought to contribute to the pathology of several neuropsychiatric disorders. Here, the neuroanatomical, developmental, and physiological aspects of the lateral habenula and the fasciculus retroflexus will be discussed, as well as its implications in depression and schizophrenia.

Keywords: Lateral habenula; Fasciculus retroflexus; Development, Axon guidance molecules; Dopamine; Serotonin; Reward; Learning; Memory; Attention; Depression; Schizophrenia

Introduction

The dorsal diencephalic conduction system (DDC) is one of the two major pathways that interconnect the limbic and striatal forebrain with areas of the mid- and hindbrain. The DDC comprises of three core components: the habenular nucleus, the stria medullaris, and the fasciculus retroflexus. In many vertebrates, the habenular nuclear complex is divided into a medial (MHb) and lateral (LHb) nucleus. It receives input from forebrain structures via the stria medullaris and exerts influence on certain midbrain cell groups via the fasciculus retroflexus (Sutherland, 1982; Bianco and Wilson, 2009). This highly evolutionary conserved DDC circuit is implicated in a diverse range of behaviors. The habenula plays a role in functions as diverse as maternal behavior, pain, sleep, stress, learning, and reward, but its exact role in most of these functions remains uncertain (Lecourtier and Kelly, 2007). In addition to these behaviors, recent studies suggest that the lateral habenula plays a pivotal role in controlling cognitive behavior by influencing the dopamine and serotonin neurons in the midbrain (Lecourtier and Kelly, 2007; Hikosaka *et al.*, 2008). Lateral habenula pathology is thought to contribute to the dysfunction of these cognitive behaviors and therefore be implicated in several neuropsychiatric disorders, such as depression, schizophrenia, and drug-induced psychosis (Ellison, 1994, 2002; Bianco and Wilson, 2009).

This thesis will review different characteristics, such as anatomy, development, and function, of the lateral habenula and the fascicules retroflexus. Particularly, it will focus on the influence of the lateral habenula on the reward system, as well as its role in several cognitive behaviors, namely, learning, memory, and attention. Finally, the implications of the lateral habenula in neuropsychiatric disorders will be discussed. Several studies have shown that dysfunction of the habenula in the dopaminergic and serotonergic systems can result in depression- and schizophrenia-like symptoms (Morris *et al.*, 1999; Lecourtier *et al.*, 2004; Ellison, 1994, 2002; Lecourtier and Kelly 2005).

This review presents a clear overview of the most crucial research concerning the role of the lateral habenula in different behaviors that seem to be impaired in depressed and schizophrenic patients. Furthermore, this will be the first paper to combine information about the development of he habenula and the fasciculus retroflexus with its functions. While comparing all the essential data from the literature, this paper hopes to shine new light on the importance of the lateral habenula in reward processing, learning, and attention. Moreover, this paper may provide additional insights into the mechanisms of these behaviors and how dysfunction of these systems may contributes to the pathology of neuropsychiatric disorders.

Chapter 1: Anatomy

The dorsal diencephalic conduction (DDC) system is one of the two major pathways that interconnects the limbic forebrain with sites in the mid- and hindbrain. The other, more ventral, pathway is the medial forebrain bundle (MFB) which originates in the anterior olfactory areas and passes through the lateral preoptic, lateral hypothalamic, and ventral tegmental areas (Sutherland, 1982). The DDC consists of three major components: the habenular nuclei (Hb); the stria medullaris (SM), which is the main fiber tract through which input from the forebrain arrives at the habenula; and the fasciculus retroflexus (FR), a prominent fiber tract that primarily carries efferent axons from the habenula towards the targets in the mid- and hindbrain (Sutherland, 1982; Bianco and Wilson, 2009). It has been hypothesized that the two pathways act parallel. Both pathways are depicted in Figure 1C. This chapter will focus on the anatomy of the different parts of the dorsal diencephalic conduction system, with the habenula (and its patterns of connectivity) in particular.

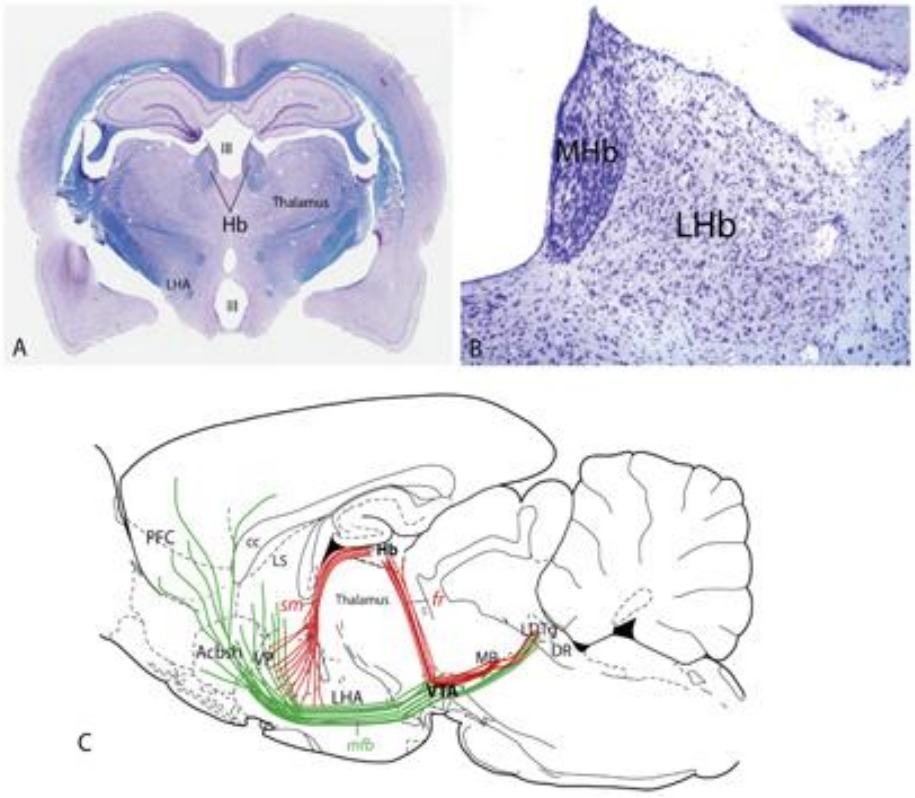

Figure 1: The habenula is situated in the dorsal diencephalon on both sides of the third ventricle (A) and consists of a MHb and a LHb (B). The Hb is part of the dorsal diencephalon conduction system that conveys information from several forebrain nuclei to ascending brainstem nuclei (C: red lines). The DDC is suggested to act in parallel to the more ventral located medial forebrain bundle (C: green lines). Figure adapted from Geisler and Trimble (2008).

1.1 The lateral habenula

The habenular nucleus is a point of convergence for neural information from the basal ganglia and limbic forebrain. From here, the information is passed on to regulatory monoaminergic mid- and hindbrain areas. In the next paragraphs, the anatomy of the lateral part of the habenula will be discussed, followed by its afferent connections, and lastly its efferent connections.

Invertebrates

Phylogenetically, the three main structures of the DDC are highly conserved and are distinguishable in all vertebrates. In fishes and amphibians, the habenular nuclei and the fasciculus retroflexus have a bilateral asymmetry. The habenulae display left-right (LR) differences in size, cytoarchitectonic organization, neurochemistry, and connectivity. In these species the right habenula is considerably larger than the left. Furthermore, the larger right habenula is linked to a thicker right fasciculus retroflexus. These asymmetries are associated with pronounced asymmetry in afferent projections from the pineal eye organs. Habenular asymmetries are significantly more subtle in birds and mammals, although there are small LR differences in size. This is partly due to the fact that birds and mammals lack pineal eyes and pineal efferents projecting to the lateral habenula and are therefore less asymmetric (Bianco and Wilson, 2009).

Vertebrates

The habenula is located above the thalamus, adjacent to the third ventricle, with its posterior end close to the midline (see Figure 1A and 1B). It is called the epithalamus together with the pineal body.

The habenular complex comprises two separate nuclei on each side and a habenular commisure connects the nucleus on one side of the diencephalon with that on the other side. In mammals, the habenular complex is composed of two separate nuclei on each side: a medial (MHb) and a lateral (LHb) nucleus. The MHb and LHb are very distinct from one another, from anatomical, as well as physiological perspectives. Both the lateral and the medial nucleus consist of smaller nuclei, ten and five subnuclei respectively (Sutherland, 1982; Bianco and Wilson, 2009). This paper will focus on the LHb, therefore only the anatomy and connectivity of the LHb will be discussed.

Subnuclear organization of the lateral habenula

The view that the habenula is not a single homogenous structure, but consists of two separate nuclei was first proposed by Nissl in 1889. He divided the habenula into the medial and lateral nuclei, based on cytoarchitectonic differences he observed among the neurons. Since then, several studies have shown that neurons of these two nuclei possess different morphological and electrical properties and are connected with different brain areas (Kim, 2009). The division into a medial and lateral nucleus is now generally accepted. After the habenula was divided into these subnuclei, the question raised whether these nuclei could be further divided into subnuclei. Already in 1977, Herkenham and Nauta conducted a tracing study with horseradish peroxidase (HRP) in order to examine the afferent connections of the habenular complex. In this study a medial (suggested to be limbic) and a lateral (suggested to be motor) area of the LHb have been described (Herkenham and Nauta, 1977). The LHb was considered to composese of this medial and lateral part for a long time. A higher heterogeneous organization of this nuclear group was not recognized until the study of Andres *et al.* in 1999. This study showed that the lateral nucleus can be further divided into ten subnuclei, 5 within its medial (LHbM) and another 5 within its lateral (LHbL) division. Via new techniques using semithin sections, Andres *et al.* developed new morphologic criteria which made the differentiation possible (Andres *et al.*, 1999). The medial subnuclei of the lateral habenula (LHbM) are distinguished from the LHb by its composition of smaller nerve cells, a light neurophil, and its scarceness in myelinated fiber bundles. Furthermore, the formation of the lateral root of the fasciculus retroflexus consists of a large proportion of unmyelinated axons and small myelinated fibers. Concerning the characteristic dendritic formations, coarse or fine neuropil, and differentially sized and shaped neurons, several nuclei have been distinguished in the LHbM. The anterior part (LHbMA) is the most rostral

extension of the LHb. The superior part (LHbMS) is recognized by its relatively large dendrites. The parvocellular part (LHbMPc) usually is found just below the LHbMS, but these spindle-shaped neurons are smaller compared with those in the LHbMS. The central part (LHbMC) is situated between the LHbMPc and the LHbL. Lastly, the marginal part (LHbMMg) is the shortest of the LHbM subnuclei.
The LHbL is located more caudally compared with the LHbM and contains mostly larger neurons. It includes a substantial number of fibers, which form the lateral root of the fasciculus retroflexus. Just as the medial part of the lateral habenula, the lateral part was divided into five subnuclei. The magnocellular part (LHbLMc) is the most prominent subnucleus of the LHbL. It contains especially large nerve cells and thick, long dendrites. The basal part (LHbLB) forms the ventral border of the LHbL. The oval part (LHbLO) appears as an oval field in the lateral extension of the LHbLMc. The parvocellular part (LHbLPc) forms the dorsal border of the LHbL, it is positioned just below the lateral stria medullaris. Lastly, the marginal part (LHbLMg) is situated at the most dorsal edge of the LHbL, even dorsal to the fibers of the lateral stria medullaris. Andres *et al.* (1999) made graphic reconstructions in order to get a three-dimensional impression of the arrangement and size of all the subnuclei of the lateral habenular complex. These graphic reconstructions are depicted in Figure 2.

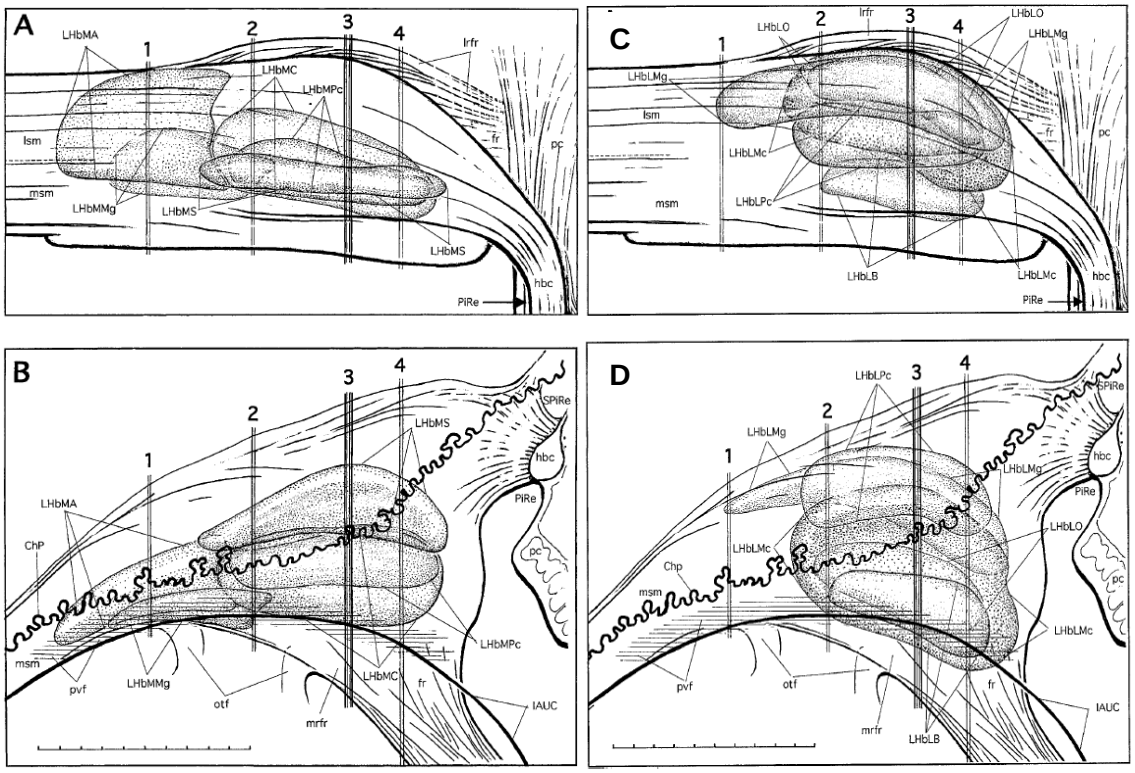

Figure 2: Graphic representation of the arrangement and size of all the subnuclei of the lateral habenular complex. A and B represent the LHbM, while C and D represent the LHbL. A and C are a dorsal view, B en D are a view from the median face. Scalebar = 1mm. Figure adapted from Andres *et al.* (1999).

These findings could have very important consequences. Recognizing borders and defining intranuclear heterogeneities are basics for defining the morphologic organization of brain nuclei as a first step towards understanding their biological functions (Andres *et al.*, 1999; Geisler *et al.*, 2003). After primary identification, however, additional information on the chemoarchitecture, the connections, and possibly a distinct function, is required to determine that a morphologically defined structure represents a functional subnucleus (Geisler *et al.*, 2003). To gain further independent information, more experiments were conducted by

Geisler *et al.* (2003) to try to detect the different subnuclei using different criteria. For instance, by using immunocytochemical visualization of a panel of selected neuroactive substances (Geisler *et al.*, 2003).
Still, it remains to be elucidated whether these subfields in fact constitute separate subnuclei that are morphologically and functionally distinguishable from one another (Kim, 2009).

The anatomical organization of the dorsal route can be described most concisely in terms of afferent to and efferent connections from the two habenular nuclei. This paper will only focus on the lateral nucleus and therefore only the most important afferent and efferent connections of this nucleus will be discussed.

1.2 Afferent connectivity

The lateral habenula is a point of convergence for neuronal information from the basal ganglia and limbic forebrain. Afferent fibers reach the lateral habenula via the stria medullaris, the fasciculus retroflexus, and the habenular commissure. Primarily the fibers of the lateral part of the stria medullaris terminate in the lateral habenula (Sutherland, 1982).
As described above, the lateral habenula can be subdivided into lateral and medial subnuclei. The medial subnuclei receive afferents primarily from limbic brain regions that are directly or indirectly innervated by the cerebral cortex. While the lateral division is mainly innervated by the basal ganglia, in particular the globus pallidus internus, and the limbic system (Hikosaka *et al.*, 2008). An overview of the afferents and efferents of the habenular complex is shown in Figure 3.

Basal ganglia
The major source of innervations of the LHb of the rat is the entopeduncular nucleus (EPN). This entopeduncular nucleus is the non-primate homologue of the internal segment of the globus pallidus (part of basal ganglia). HRP-tracing studies in rats showed that virtually every entopeduncular neuron appears to project to the LHb (Herkenham and Nauta, 1977). This pallid-habenular pathway has also been demonstrated in cats and monkeys. The entopeduncular projection appears to be topographically organized within the lateral nucleus. Medially pallid-habenular neurons merge with a large population of lateral hypothalamic neurons that also project to the LHb. Therefore, the habenular nucleus appears to be a major convergence point of projections from these otherwise quite separate cell regions.
Moreover, the LHb receives a strong input from the ventral pallidum which contributes to the ventral basal ganglia loops and is densely innervated by the nucleus accumbens (NAcc).

Limbic system
Limbic regions of the forebrain constitute the second major source of afferent innervations to the LHb. A continuous band of cells stretching from the anterior preoptic area, through the lateral hypothalamus, to the mid-hypothalamus, projects to the LHb. Comparable to the entopeduncular projections, a medial-lateral topographic organization has been described for the projections from the lateral preoptic and lateral hypothalamic areas (Herkenham and Nauta, 1977).

Ascending input
The ventral tegmental area (VTA), median raphe, and ventral central grey give rise to ascending input to the lateral habenular nucleus. The contributing afferents from the midline region of the VTA (interfascicular and paranigral nuclei) probably distribute input via the

fasciculus retroflexus. This projection provides the habenular complex with the major part of its dopaminergic innervations.

Thus, a unique feature of the afferent sources of the lateral habenula is the convergence of limbic efferents, composed of septal, lateral preotic, and lateral hypothalamic fibers, with striatal efferents, composed of entopeduncular fibers and fibers from the ventral pallidum. The major part of the dopaminergic input to the lateral habenula constitutes of ascending input from afferents of the VTA (reviewed by Sutherland, 1982; Bianco and Wilson, 2009).

1.3 Efferent connectivity

The efferents of the lateral habenula are extensively distributed and establish efferent connectivity with a wide range of targets (Araki *et al.*, 1988). Many of these targets are themselves sources of afferent input to the LHb, for instance, the earlier mentioned VTA (Lecourtier and Kelly, 2007). The fasciculus retroflexus is an important fiber tract that mostly carries the efferent axons from the habenula towards the targets in the min- and hindbrain. All of the LHb efferents enter the fasciculus retroflexus and compose the mantle portion of the bundle. The core of this bundle comprises the efferents from the MHb. The fasciculus retroflexus arises from the posterior lateral aspect of the habenula and courses downwards across the junction of the diencephalon and the tectum toward the interpeduncular nucleus (IPN) on the ventral side of the midbrain. Before the FR reaches the IPN, several axons split from the FR and turn to other midbrain nuclei (Kim *et al.*, 2005). An important part of the efferent fibers from the LHb innervate the raphe nucleus and the VTA, therefore these efferents will be discussed in the next paragraphs. A complete overview of the afferents and efferents of the habenular complex is shown in Figure 3.

Raphe nucleus
The main output of the LHb is directed caudalward, targeting midbrain serotonergic, dopaminergic, and cholinergic neuronal groups (Herkenham and Nauta, 1979).
The LHb initiates descending connectivity to numerous monoaminergic nuclei in the mid- and hindbrain. One major projection innervates the dorsal and median raphe nucleus; this has been well established by studies using radioactively-labelled amino acid- and retrograde HRP transport. Using these techniques additional descending efferents to the substantia nigra pars compacta, superior colliculus, and central grey have been described. The substantia nigra pars compacta projects to the dorsal striatum, which in turn connect to the pallidum, a major source of afferent innervation to the LHb.

VTA
A second major descending projection of the LHb terminates in the ventral tegmental area (Araki *et al.*, 1988). In the VTA, LHb efferents become organized into groups that disperse in several directions. There are some roots for feedback in this circuit: the VTA also projects directly to the LHb and to the nucleus accumbens, which is a source of LHb afferent innervation.
Next to this major descending projection, the LHb projects strongly to a recently identified cell group in the ventromedial mesopontine tegmentum (tVTA/RMTg) situated behind the VTA and dorsolateral to the interpeduncular nucleus (Jhou *et al.*, 2009). This is of interest because this small nucleus projects to the entire midbrain dopaminergic system (Geisler and Trimble, 2008).
In addition to the aforementioned descending efferents, there are also ascending efferents. These ascending efferents mainly project to the dorsomedial and anterior nuclei of the

thalamus, dorsomedial and lateral hypothalamus, lateral preoptic area, substantia innominata, and lateral dorsal tegmental nucleus (reviewed by Sutherland, 1982; Bianco and Wilson, 2009).

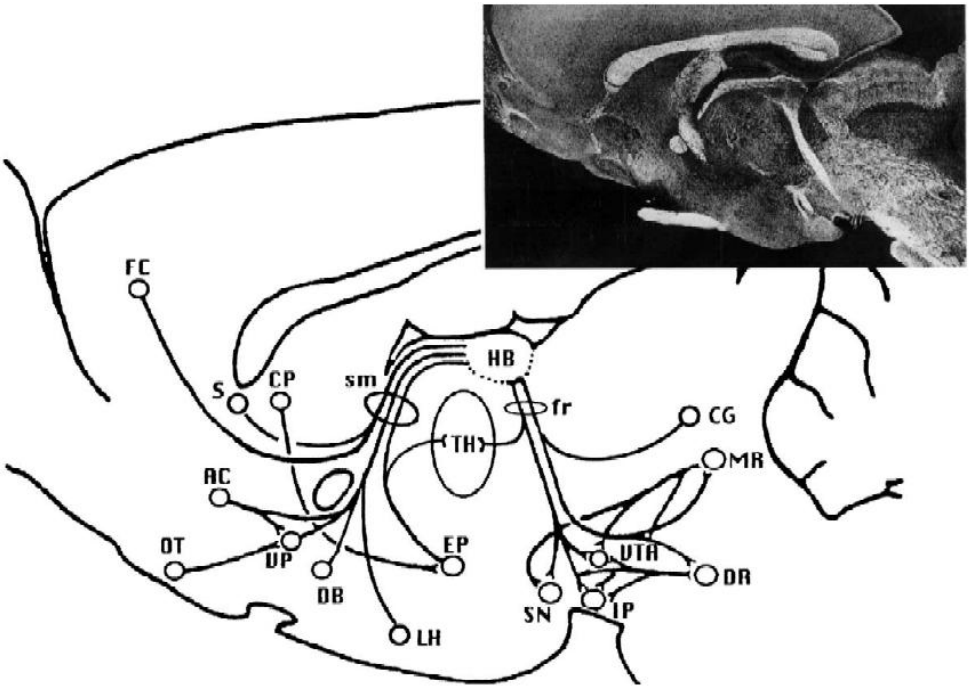

Figure 3: Schematic representation of some of the primary inputs and outputs of the habenular copmplex. Insert shows saggital section with myelin stain, demonstrating the FR. The two major tracts are the SM, ascending into the habenula, and the FR, descending from habenula. For abbreviations: see abbreviations list. Figure adapted from Ellison (1994, 2002)

Chapter 2: The development of the habenula and the fasciculus retroflexus

During the development of the central nervous system, growing axons reach their targets via very specific routes, resulting in a correct, complex wiring of neuronal circuits. Proper functioning of the nervous system requires precision in wiring structures together. Pathfinding depends by most axons upon a complex combination of attractants and repellents (guidance cues) in the surrounding environment. Growth cones, the hand-like structures at the end of growing neurites, have an important role in detecting directional cues. The growth cones can be influenced and thereby guided by secreted or contact-mediated attractive and repulsive signals in the environment (Figure 4) (Mueller, 1999; de Castro, 2003). Several families of guidance molecules have been identified. These include the netrins, ephrins, slits, and semaphorins (Giger *et al.*, 2000). These guidance cues are multifunctional; they can either act as a repellant or as an attractant. Moreover, these guidance molecules do not act in isolation to define the routes taken by the developing axons. The combination of guidance cues together with intrinsic and extrinsic factors regulates the responsiveness of growth cones. Together, these mechanisms cause diversity in growth cone behavior (Dickson, 2002).

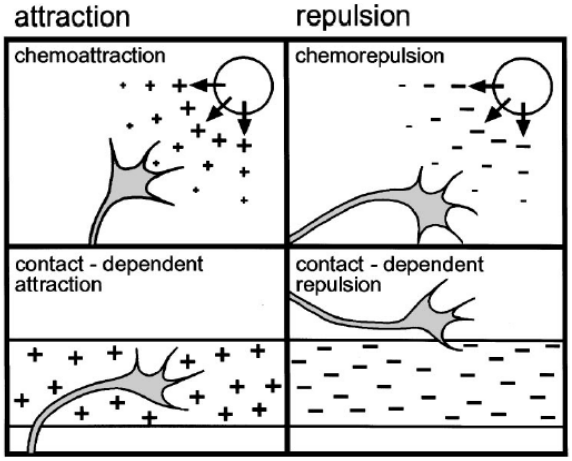

Figure 4: Growth cone guidance mechanisms. Guidance mechanisms are classified as being either attractive or repulsive, with both acting over long (chemo-attraction and -repulsion) or short range distances (contact-dependent attraction or repulsion). Figure adapted from Mueller (1999).

These axon guidance molecules will also be used for the development of the axons from the habenula to the midbrain structures, such as the interpeduncular nucleus, the VTA, and raphe nucleus. Since the habenula projects to various brain areas, it influences many different functions, these functions of the habenula and its projections to these monoaminergic midbrain structures will be discussed in chapter 3. To obtain a better understanding of the different functions, it is also important to investigate the development of the habenula and the fascicules retroflexus. Therefore, this chapter will discuss the most prominent research to the development of the habenula and the fasciculus retroflexus and the axon guidance molecules that are necessary to realize this important tract.

2.1 Genes important for habenula development

Before elaborating on the development of the fasciculus retroflexus, this paragraph will briefly discuss the development of the habenular neurons itself. Unfortunately, only little is known about the development of the habenula in mammals.
In fishes and amphibians, the habenula develops bilaterally asymmetrical in relationship with the photoreceptive pineal organ. In the zebrafish diencephalon, asymmetric expression of Neuropilin1a (Nrp1a) mediates the difference in connectivity between the left and the right habenular efferents (Kuan *et al.*, 2007). However, mammals lack the pineal organ and therefore also the asymmetry appears to be lost (reviewed by Bianco and Wilson, 2009).
Recently, one study by Quina *et al.* (2009) demonstrated a role for the POU-domain transcription factor Brn3a (product of *Pou4f1* gene) in habenula development. Microarray

analyses showed that Brn3a is required for the coordinated expression of a battery of genes that characterize the habenula. Most of these genes are highly enriched in the habenula and include transcription factors, components of neurotransmitter systems, and K^+ and Ca^{2+} channels. Nearly all the MHb neurons and a part of those in the LHb, express the transcription factor Brn3a. These neurons exclusively project, via the FR, to the IPN and do not contribute to the earlier described LHb projections to the midbrain monoaminergic nuclei. Hereby, this study identifies a set of neurons in the LHb which shares gene expression and connectivity characteristics with the MHb. *Pou4f1*$^{-/-}$ embryos initially have an appropriate FR tract, but the axons fail to connect to their target, the IPN. Therefore, Brn3a is required for correct innervation of habenular targets. In the habenula, Brn3a appears to function only as a transcription factor (Quina *et al.*, 2009). However, it remains largely unknown how these Brn3a transcripts contribute to the habenula development. Since *Pou4f1*$^{-/-}$ embryos develop an appropriate FR, but the axons fail to connect to their target, Brn3a transcripts could be genes involved in this innervation process. The formation of synapses onto targets is a very complex process and involves many different proteins that act from a distance or at site (reviewed by Waites *et al.*, 2005). Thought, it would be possible that Brn3a is a transcription factor for genes that produce proteins important for this process of synaptogenesis. More research into the Brn3a transcripts is necessary to identify their downstream targets and their function in the developmental process of the habenula and the FR.

2.2 Segmental organization

The central nervous system develops segmentally along the rostral-caudal axis to form neuromeres separated by transverse constrictions in the forebrain and hindbrain. The forebrain is divided into six transverse domains, named prosomeres. These prosomeres can be grouped into two large transverse subdivision: the diencephalon and the prosencephalon. The diencephalon is considered to comprise of the first three prosomeres (p1, p2, and p3) and four neuromeres (D1, D2, D3, and D4) (Rubenstein *et al.*, 1994). Circumferential axonal tracts are formed along the neuromere boundary regions (Puelles and Rubenstein, 2003). This suggests that early pioneer axons make effective use of guidance cues expressed within these segments (prosomeres) to establish a simple axon scaffold upon which following generations of axons selectively fasciculate and extend. Two different guidance cues are thought to regulate this axonal growth between prosomeres: repulsive cues expressed within the prosomeres that prevent the axon from crossing into these areas and attractive cues expressed on the initial established axon that promote the fasciculation of the following axons (Figdor and Stern, 1993). The fasciculus retroflexus is formed along the boundary between prosomere 1 and prosomere 2. Although the exact molecular mechanisms for this formation is still puzzling, it is thought that repulsive axon guidance molecules are expressed in the adjacent neuromere or that each boundary region expresses different molecules that are able to guide the axonal growth (Funato *et al.*, 2000).

2.3 Axon guidance events underlying FR development

The floor plate at the ventral midline of the neural tube has a pivotal role in directing circumferential axonal growth in the hindbrain and spinal cord. Netrin-1, secreted from the floor plate is thought to mediate the attractive guidance signal for this axonal growth (Shirasaki *et al.*, 1995). Furthermore, DCC (deleted in colorectal cancer) acts as an attractive receptor for netrin-1 (Keino-Masu *et al.*, 1996). This attractive signal of netrin-1, in combination with repulsive signals from the semaphorins, Sema3F and Sema5A, is thought to contribute to the growth of the fasciculus retroflexus by guiding it along the neuromere

boundary region (Funato *et al.*, 2000). In the next paragraphs these axon guidance molecules and their function in the development of the fasciculus retroflexus will be discussed in more detail.

Semaphorin family

Among the earlier mentioned families of guidance molecules, the semaphorin family is the largest family of repulsive and attractive growth cone guidance proteins. Two receptor families have been implicated in mediating many semaphorin functions: plexins and neuropilins. Many classes of semaphorins have shown to interact directly with plexins, however, class 3 secreted semaphorins specifically binds to receptors from the neuropilin protein family. Neuropilins, though, are not sufficient to induce a guidance signal mediated by class 3 semaphorins. An intermediate molecule is necessary to produce guidance activity. Proteins of the plexin family are thought to bind to neuropilins to form a receptor complex for class 3 semaphorins (reviewed by Pasterkamp and Kolodkin, 2003).

Development of the habenular nucleus and the fasciculus retroflexus

Lateral habenula neurons of the rat are developed at embryonic day 12 (E12) through E15, with a peak at E13 and E14 (Altman and Bayer, 1979). One important study by Funato *et al.* (2000) investigated the development of the fasciculus retroflexus. Using different stainings, they demonstrated that a few axons of the FR reached the ventral midline and then turned caudally at E14. As seen in Figure 5, the FR left the habenular nucleus as a tight bundle and was defasciculated in the ventral region. At E13, a group of growing axons from the habenular nucleus was observed along the neuromere boundary, while at E12 no axonal tract was visible in this region. Therefore, the initial neuronal growth from the habenular nucleus (the FR) occurs between E12 and E13 (Funato *et al.*, 2000). In addition, this study confirms the earlier results of Altman and Bayer (1979) that the first population of the habenular neurons was formed at E12. Since the attractant netrin-1 was expressed at E12 in the ventral region of the diencephalon, the habenular neurons began to differentiate and extend their axons more ventrally between E12 and E13.

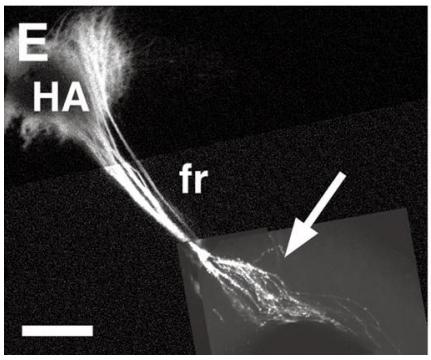

Figure 5: Migrating axons of the FR reach the ventral midline region of the caudal diencephalon (arrow). Left is rostral and top is dorsal. Figure adapted from results section Funato *et al.* (2000)

Netrin-1

Funato *et al.* (2000) showed that netrin-1 was capable of attracting habenular neurons. Moreover, the attractive receptor for netrin-1, DCC, was expressed in the FR at E14. The receptor DCC seemed to be necessary for the FR axons to grow towards the netrin-1 expressing cells in the ventral region, as addition of an antibody for DCC in cultured FR axons blocked the axonal outgrowth of the FR neurons (Funato *et al.*, 2000). The role of DCC in this axonal growth has been confirmed by other studies. Shu *et al.* (2000) also found that DCC is expressed in the developing habenular commissure, the FR, and the stria medularis at E13 and E14 (Shu *et al.*, 2000).

Neuropilin-2-Sema3F

Next, neuropilin-2 was expressed in the habenular neurons, although the expression in the lateral habenula differed from that in the medial habenula. Whereas Neuropilin-2 expression

in the lateral habenular nucleus was observed at E13 through E15, decreased after E15, and was not detectable after E19. Neuropilin-2 expression in the medial habenular nucleus was generated between E14 and E18, but was observed from E16 through adult stage. Funato *et al.* (2000) hypothesizes that this difference in neuropilin-2 expression in the lateral and medial habenular nuclei may explain the different projection patterns of the habenular nuclei.
In addition to the neuropilin-2 expression in the habenular neurons at E13-E14, there was a strong expression of Sema3F in the developing diencephalon. This expression was limited to the rostral p1, adjacent to the developing FR which arrived from the habenular nucleus expressing neuropilin-2. Through this Sema3F-neuropilin-2 interaction, a repulsive signal is transmitted and thereby regulates the axonal growth of the FR.
As discussed above, neuropilins are not sufficient to induce axon guidance activity via its bindings to the class 3 semaphorins. Proteins of the plexin family are thought to bind to the neuropilins en thereby forming a receptor complex. Neuropilin-2 has been coprecipitated with plexin-A1 and –A3 (Takahashi *et al.*, 1999). In addition, plexin-A1 and -A3 are expressed broadly in the developing diencephalon, including the habenular nucleus at E14. This suggests that both plexin proteins can bind to neuropilin-2 in the FR in order to form a functional receptor for Sema3F (Funato *et al.*, 2000). In addition to these results, other studies showed that neuropilin-2 deficient mice show a defective FR (Chen *et al.*, 2000; Giger *et al.*, 2000), which confirms the importance of neuropilin-2 for the proper axonal growth of the FR.

Sema5A

As described above, axons from the habenular nucleus pioneer the FR beginning around E13 in rats. Another semaphorin molecule, Sema5A, is expressed in two distinct locations in the diencephalon during this developmental period. At E13.5, Sema5A transcript is expressed in the habenular nucleus itself and at E15.5, Sema5A colocalizes with neuropilin-2. Moreover, Sema5A is expressed in prosomere 2 at E15.5 and tightly surrounds the FR as it projects ventrally. In diencephalic tissues without Sema5A, the FR neurons were no longer restricted to the boundary between p1 and p2; they were more likely to cross into prosomere 2. Furthermore, the FR axons often did not reach their target in the ventral midbrain. These results suggest that this member of the semaphoring family also appears to play an essential role for a proper FR development (Kantor *et al.*, 2004).

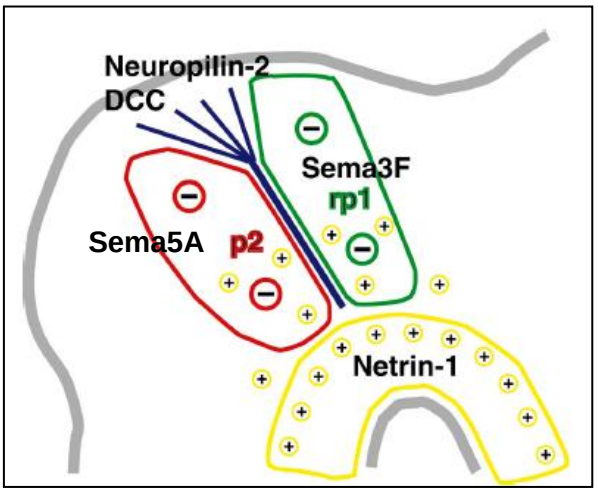

Figure 6: Scheme of circumferential growth of habenular axons along the neuromere boundary. Fr (blue line) originates from the habenular nucleus expressing *neuropilin-2* and DCC. FR is attracted to *netrin-1* secreted from the ventral region of the diencephalon (yellow area) and repulsed by the rostral p1 expressing Sema3F (green area). In addition, p2 (red area) is also repulsive to the FR through Sema5A. The combination of these area-specific signals is thought to guide the axons to form a thick bundle along the boundary region. Figure adapted from Funato *et al.* (2000)

As mentioned earlier, intermediate molecules can be necessary for proper guidance activity of the semaphorin axon guidance molecules. Sema5A is a bifunctional guidance cue regulated by sulfated proteoglycans. The permissive effects of Sema5A on the habenular axons result from interactions with axonally expressed heparan sulfate proteoglycans (HSPGs), while the inhibitory effects depend on interactions with chondroitin

sulfate proteoglycans (CSPGs). Thus, depending on the types of sulfated proteoglycans in the developmental environment, the growth cones can react in a different manner to Sema5A. Sema5A expressed on the FR axons is likely to promote the highly fasciculated morphology of the FR through interactions with neuronally expressed HSPGs. Furthermore, both Sema5A and CSPGs are well positioned in the developing diencephalon. Together they create an inhibitory signal to prevent the developing axons of the FR to cross the p2 border (Kantor *et al.*, 2004).

In conclusion, the combination of a repulsive signal from rostral p1 (Neuropilin-2-Sema3F), another repulsive signal from p2 (Sema5A-CSPGs), and an attractive signal from netrin-1 at the ventral side, in combination with the expressed DCC on the developing axons, contributes to the linear and fasciculated growth of the habenular axons along the boundary region (Figure 6) (Kantor *et al.*, 2004; Funato *et al.*, 2000).

2.4 Future perspectives

To fully understand the functions of the habenular complex, it is very important to gain a better understanding of the development of the habenula and its efferents. Since the habenula projects to many different brain areas, it is implicated in a variety of functions. How these different connections been realized is important for the understanding of the different functions. Still, only a little is known about the development of the habenular nuclei. Quina *et al.* (2009) demonstrated that the transcription factor Brn3a plays a crucial role in the development of the habenular neurons. They defined a unique habenular profile of neurotransmitters, receptors, ion channels, and regulatory factors. Knock-out mice of the Brn3a gene develop a proper FR, but it does not innervate its target (Quina *et al.*, 2009). More research into the transcripts of Brn3a is necessary in order to understand its role in the developmental process.

The MHb and LHb project to different brain areas. Besides, axons from only the MHb or the LHb project to a variety of different brain areas. For several important guiding cues it is known how they guide the pioneer axons from the habenula towards the ventral side of the diencephalons. Kantor *et al.* (2004) and Funato *et al.* (2000) created a model for the development of the fasciculus retroflexus. A few pivotal axon guidance cues have been discovered to be crucial for the correct guidance of the FR.
In a recent study by Dugan *et al.* (2011) Slit/Robo signaling molecules showed to play an important role in maintaining proper dorsoventral positioning, promoting straight axonal growth through the diencephalon, and preventing ectopic projections into the ventral midline. These results were shown to be crucial for a proper trajectory of the ventral mesodiencephalic dopaminergic (mdDA) axons (Dugan *et al.* 2011). Therefore, one could assume that these signaling molecules would also prevent the FR from ectopic projections into the floor plate. More research on these signaling molecules in relation to the FR is needed.

The axons from the LHb comprise the sheaths of the FR, while the core of the FR contains the axons from the MHb (Kim *et al.*, 2005). How do these differences in core/sheath division of the FR develop? Do these axons of the FR develop different over time? Do they develop at the same time, but are there different guidance cues responsible for the core/sheath differentiation? How are the axons form the core of the FR guided towards different target areas than the axons from the sheath of the FR? These are interesting research questions that remain to be investigated. It would also be interesting to research the kind of connections that are realized in the different target areas. In order to understand the role of the projections to

the target areas, it is important to what kind of projections these are and how they are established. More research is needed in order to understand the complete developmental process of the habenula and its projection through the FR to the different target areas.

Chapter 3: Functions of the habenular complex

The habenular complex and the fasciculus retroflexus exert influence upon many different behaviors. These behaviors include the regulation of sleep, reward, pain, sexual and maternal behaviors, and anxiety. Recently, studies shown that the habenula also plays a role in cognitive behavior, for example, learning, memory, and attention (Lecourtier and Kelly, 2007).

Several recent reports provide evidence for strong functional associations between the LHb and the dopaminergic neurons in the ventral midbrain, which are involved in adjusting motor behaviors and learning new behavioral responses to salient stimuli (Bianco and Wilson, 2009). In addition, the fact that the habenula receives a strong input from the entopeduncular nucleus (see chapter 1), which is the output structure of the basal ganglia, indicates that the LHb is also involved in motor, motivational, and rewarding systems (Lecourtier and Kelly, 2007). Moreover, the involvement of the lateral habenula in spatial learning and memory is proposed by studies in which levels of brain metabolic activity have been associated with the performance in memory tasks and studies in which the effect of habenular lesions on memory is investigated. In support of this notion, fMRI studies have been conducted on human subjects, normally and cognitively impaired groups, while performing cognitive tasks. Evidence from these studies suggests an important role for the LHb and the FR in memory and attention (reviewed by Hikosaka *et al.*, 2008).
Although many neuromodulator systems are influenced by fibers of the fasciculus retroflexus, this paper focuses on the projections that influence the dopamine and serotonin systems, since these seem to be involved in many of the above mentioned behaviors, including the cognitive behaviors.
This chapter will mainly focus on the influence of the LHb in several behaviors, namely the dopamine reward system, learning and memory, and attention. Before elaborating on its implications in the reward system, first the functional relationship between the lateral habenula and dopamine neurons in the VTA and substantia nigra will be discussed. The end of this chapter will focus on the role of the LHb in learning, memory, and attention.

3.1 Projections from the lateral habenula to the VTA

The irregular firing of the VTA neurons changes to a high-frequency burst pattern in response to reward-predicting stimuli and novelty. Glutamatergic afferents play a key role in regulating this VTA cell firing (reviewed by Geisler and Wise, 2008). Activation of these afferents results in an increase in the firing rate of the dopamine neurons in this area. In addition, local infusion of glutamate receptor agonists activates the VTA dopaminergic neurons, while blocking ionotropic glutamate receptors inhibits VTA dopaminergic neurons. These findings emphasize the role of glutamate in exciting neurons in the VTA. There are many brain areas projecting glutamatergic neurons to the VTA, including the lateral habenula (Geisler *et al.*, 2007).
In contrast to the detailed knowledge on the excitation, the inhibition of the dopaminergic neurons in the VTA is less well understood. Several studies have been conducted on the projections from the lateral habenula to the VTA. Growing evidence suggests that the lateral habenula controls the inhibition of the VTA dopaminergic system via its efferents through the FR and thereby influences reward-related behaviors. Supporting evidence for this inhibition comes from lesion-studies of the habenular complex (Nishikawa et al., 1986) and electrical stimuli (Christoph et al., 1986; Ji and Shepard, 2007) of the LHb, resulting in activation and inhibition of the mesencephalic dopamine neurons, respectively.

The projections from the LHb to the VTA seem to arise mainly from the central and magnocellular subnuclei of the LHb (Brinschwitz *et al.*, 2010). Several lines of evidence showed that this output from the LHb is predominantly glutamatergic (Geisler *et al.*, 2007; Brinschwitz *et al.*, 2010). One important study by Matsuda and Fujimura (1992) demonstrated that activation of habenular efferents primarily produces an excitation in any type of VTA neurons. Moreover, this effect appeared to be mediated by the neurotransmitter glutamate. In conclusion, the habenular efferents are glutamatergic and therefore excitatory (Matsuda and Fujimura, 1992).
Since activation of habenula neurons result in inhibition of dopamine neurons in the VTA, the excitatory axons from the LHb may most likely terminate on inhibitory interneurons in the VTA. In addition, the LHb efferents could end in the tail of the VTA, also called mesopontine rostromedial tegmental nucleus (tVTA/RMTg), which provides *gamma*-aminobutyric acid (GABA)ergic projections to the VTA (Geisler and Trimble, 2008; Jhou *et al.*, 2009). Then, the excitatory input from the LHb can cause an inhibitory response on the dopaminergic VTA neurons.
Recently, some studies have been conducted to examine these glutamatergic projections from the LHb to the VTA. One of these studies showed evidence that the LHb-dependent inhibitory activity in the VTA is due to an excitatory glutamatergic projection of LHb neurons, through the FR, targeting inhibitory GABAergic neurons in the VTA or the tVTA/RMTg. Only a small portion (about 10%) of the incoming LHb fibers would terminate directly on the dopamine neurons in the VTA (Brinschwitz *et al.*, 2010). Besides, connections have been established between GABA neurons and neighboring dopamine cells in the VTA. Thus, some LHb efferents that synapse onto GABA cells in the VTA will indirect inhibit the dopamine neurons (Omelchenko and Sesack, 2009).
A second study by Omelchenko *et al.* (2009) showed that the LHb projections exhibit no overt selectivity for synapsing onto GABA- versus dopamine neurons (Omelchenko *et al.*, 2009). While these data, LHb efferents making synapses directly onto dopaminergic neurons, first appeared surprising, recent electrophysiological experiments have revealed that a few dopaminergic VTA neurons indeed displayed excitatory responses to habenular stimulation (Ji and Shepard, 2007).
Figure 7 shows a schematic diagram illustrating the projections from the LHb to the VTA. However, this relatively small circuitry (LHb - GABA(VTA) - dopamine(VTA)) does not explain how LHb stimulation could evoke inhibition in VTA dopamine neurons (Ji and Shepard, 2007). The observation of only modest synapses within the VTA suggests that the uniform inhibitory influence mediated by the LHb onto dopamine cells involves some other intermediate source of GABA. For several reasons the tVTA/RMTg is the best candidate for this intermediary. Firstly, the tVTA/RMTg receives substantial innervations from the LHb (Herkenham and Nauta, 1979; Jhou *et al.*, 2009), secondly, it contains almost exclusively GABAergic neurons (Olson and Nestler, 2007), and lastly, it has proven to project widely to the entire nigra-VTA complex (Jhou *et al.*, 2009).

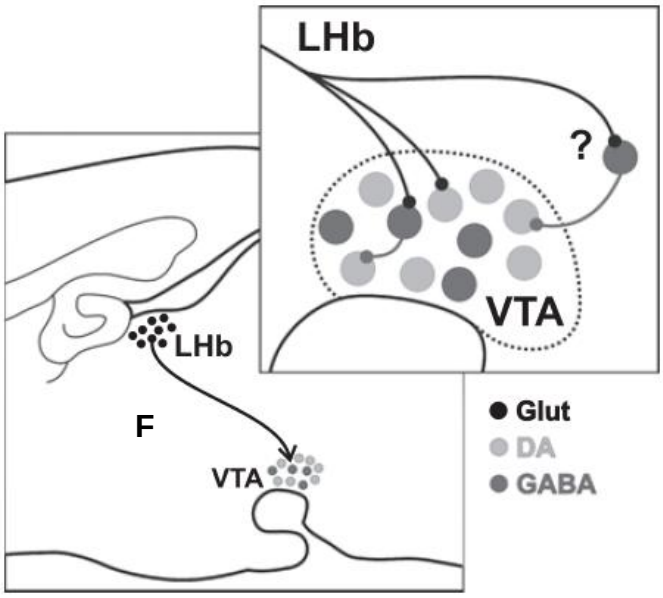

Figure 7: schematic diagram illustrating the projections from the LHb to the VTA. LHB projections, via the FR, to the VTA are predominantly glutamatergic (black) and synapse onto both dopamine (light grey) and GABA (dark grey) neurons. The latter provides collateral innervation of dopamine cells. An extrinsic source (question mark) contributes to the inhibition of the dopamine cells; this is most likely the tVTA/RMTg. Figure adapted from Omelchenko *et al.* (2009).

The presence of multiple direct and indirect pathways that connect the LHb to the VTA suggests that the overall functional impact of the LHb on the VTA is likely to be complex. Even more complex is the fact that the VTA projects directly and indirectly back to the LHb. Therefore, the LHb has a controlling effect on dopamine neurons in the VTA and substantia nigra.

The lateral habenula, which receives dopaminergic inputs from the substantia nigra and VTA (Gruber *et al.*, 2007), contains considerable D2 but fewer D1 dopamine receptors. Several limbic forebrain dopamine-rich areas are innervated by the VTA. Three of these (frontal cortex, cingulated cortex, and nucleus accumbens) project directly back to the medial sector of the LHb. The LHb projects back to the VTA, thus completing a loop. The lateral habenula receives also a direct input from the VTA, specifically from a subgroup of midline VTA neurons in the intrafascicular nucleus (IFN) and medial paranigral VTA (VTApn). This dopaminergic input to the LHb projects to the medial or "limbic" portion of the lateral nucleus (Phillipson and Pycock, 1982). If these direct projections of dopaminergic VTA neurons also travel through the fasciculus retroflexus is still unclear.

3.2 The dopamine reward system

Midbrain dopaminergic neurons are indispensable for goal directed behavior. They respond to rewards and reward-predicting stimuli. That dopamine is important for reward has been proposed in many studies. Normally, rewarding stimuli of all kinds, food, water, and several drugs of abuse, become ineffective as reward in animals given low doses of dopamine-antagonists. Furthermore, animals do not lever-press for food or water if the dopamine function is impaired (reviewed by Wise, 2004). Animal models have demonstrated the importance of the VTA and its dopaminergic projections to the nucleus accumbens, limbic, and frontal structures (Figure 8) for reward and exploratory behavior.

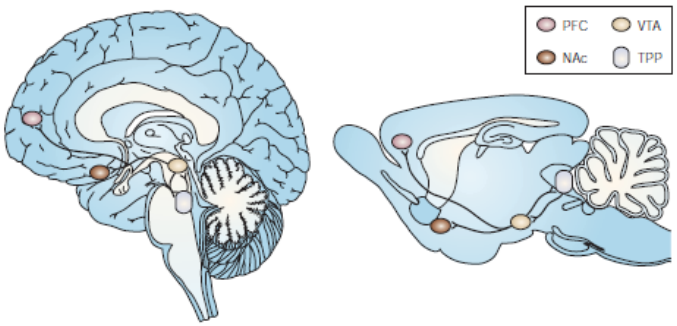

Figure 8: Human (left) and rat (right) brains, showing the mesolimbic and mesocortical dopamine pathways, which originate in the VTA and send ascending projections to the nucleus accumbens (NAc) and prefrontal cortex (PFC), respectively.

Thus, firing of midbrain dopamine neurons is essential for reward learning. The firing increases when an unexpected reward occurs, if however, a fully predicted reward was withheld, a transient cessation in spontaneous firing occurred precisely at the time the reward would have been delivered. These results indicate that dopamine neurons program an error signal that reports the difference between observed and expected events (reviewed by Schultz, 2006). Moreover, one could suggest that the brain dopamine is more responsive to the predictors of reward than to the reward itself (reviewed by Wise, 2004). Several recent studies have suggested that the lateral habenula may have an important influence in the reward-related dopamine cell activity (Shepard *et al.*, 2006).

The role of the lateral habenula projections in dopamine reward processing.

As described above, the lateral habenula projections to the midbrain are able to alter the activity of dopamine neurons. Lesions of the LHb or FR result in an increased dopamine release in the nucleus accumbens, frontal cortex, and striatum, suggesting that the LHb has an inhibitory influence on the midbrain dopamine neurons. Since dopamine plays an important role in reward-related behavior, the hypothesis that the habenula has a function in the reward system is very likely. Several studies, both animal and human, have been conducted in order to investigate this hypothesis.

The pivotal role of the lateral habenula in encoding negative outcomes for the reward system has recently been demonstrated in a study comparing the activity of habenula neurons and dopamine neurons in behaving primates. This study showed that in unrewarded trials, the excitatory response of habenula neurons started earlier than the inhibitory response of the dopamine neurons; in rewarded trials, however, the excitatory response of the dopamine neurons started earlier than the inhibitory response of the habenula neurons. Thus, the excitation of habenula neurons could inhibit dopamine neurons in unrewarded trials, but inhibiting habenula neurons could not initiate the excitation of dopamine neurons in rewarded trials. The pattern of LHb cell activity is the inverse of dopamine neurons firing under the same conditions. The investigators of this study suggest that the lateral habenula is capable of producing the negative reward response of dopamine neurons. Therefore, it appears that the LHb provides information regarding the nature of salient environmental stimuli to the midbrain reward circuits in the form of negative reward-related signals (Matsumoto and Hikosaka, 2007). Thus, the habenula appears to support the neuronal trafficking necessary for an adaptive response to error, the habenula and non-dopaminergic neurons in the midbrain suppress dopamine neuron firing and generate a representation of “error” through this inhibition (Shepard *et al.*, 2006). The importance of the habenular complex in reward processing and its influence on the dopmainergic system was demonstrated in humans by Ullsperger and von Cramon in 2003. In this study they already suggest a role for the habenular complex in determining the error in reward prediction. They demonstrated that the habenula of human subjects is activated in response to the informative negative feedback that indicates behavioral errors and also when positive feedback following correct responses is omitted (Ullsperger and von Cramon, 2003). It remains however unknown how lateral

habenula neurons acquire the negative reward information. Dysfunction of this system could diminish a person's ability to learn from errors. Interestingly, this is one of the most characteristic cognitive deficits associated with schizophrenia. This will be discussed in more detail in chapter 4.

3.3 The serotonin reward system

As mentioned above, the dopamine system plays a crucial role in guiding behavior based on rewards. Many studies suggest that another monoamine neurotransmitter, serotonin, is also involved in reward processing. A prerequisite for proving this hypothesis is to show that reward related areas are strongly interconnected with serotonergic neurons. There is a great amount of anatomical evidence showing that there are major serotonergic projections to areas implicated in reward, such as the LHb, as well as projections from these sites to the raphe nuclei (reviewed by Kranz *et al.*, 2010). One important, recent study using primates provides convincing results concerning the major role of serotonin in reward-related behavior. This study of Nakamura *et al.* (2008) demonstrates that single neurons in the monkey dorsal RN encode reward information before and after the delivery of the reward. Since 30-70% of all the DRN neurons are serotonergic, depending on species, the investigators examined whether these neurons were responsible for the reward-related activity. This study indicates that the serotonergic DRN neurons modulate their activity depending on reward information. This modulation of the DRN neurons was different from those observed in dopamine neurons as a respond to reward. Whereas the dopamine neurons respond to a reward-predicting sensory stimulus, DRN neurons respond to both the reward-predicting stimuli and the reward itself. Moreover, whereas dopamine neurons only respond to a reward when this was larger or smaller then expected (they encode reward prediction error), DRN neurons respond to all the received rewards, whether or not it was expected. Finally, whereas dopamine neurons exhibit phasic responses, DRN neurons typically exhibit tonic responses (Nakamura *et al.*, 2008). More studies indicate that serotonin has a function in reward processing. There are experimental results indicating that serotonin-depleted mice show impulsive tendencies (Wogar *et al.*, 1993; Winstanley *et al.*, 2004). Furthermore, the human DRN was activated when subject learned to obtain large future rewards (Tanaka *et al.*, 2004).

The role of the lateral habenula projections in serotonin reward processing.
The reward-related responses of the DRN neurons may originate from brain areas that project to the DRN; these include dopamine neurons from the SNc, the VTA and neurons from the LHb. The dopamine neurons of the SNc and the VTA are excited as a result of a stimulus that predicts a large reward. Therefore, the DRN neurons would also be excited by the stimulus that predicts a large reward (Nakamura *et al.*, 2008). The lateral habenula exhibits a small reward preference. Thus, it is inhibited by stimuli that predict large rewards and excited by stimuli that predict small rewards (Matsumoto and Hikosaka, 2007). These changes in habenula activity would be translated in the DRN into the large-reward preference. Moreover, evidence showed that electrical stimulation of the LHb results in inhibition of dorsal raphe cell firing. Therefore, it controls the DRN activity and possibly has a direct influence on reward-related responses of the DRN (Wang and Aghajamian, 1977).

As described above, both the dopamine and the serotonin system are involved in reward-related behaviors. Dysfunctions of these systems can result in symptoms of some neuropsychiatric disorders, such as schizophrenia or depression.
For instance, anhedonia, a failure to experience rewarding stimuli, is a key characteristic of many psychiatric disorders. It is considered to be one of the negative symptoms of

schizophrenia (Hales *et al.*, 1999). Dysfunction of the reward system can cause anhedonia, the loss of a person's ability to experience rewarding stimuli. Moreover, a decreased drive to seek rewards and, consequently, mooddisorders can be due to dysfunction of the reward system. Since the LHb exerts some inhibitory control over serotonin and dopamine neurons, elevated activity of the LHb can result in a decreased drive to seek rewards. These features may contribute to depressed behavior (reviewed by Geisler and Trimble, 2008). As mentioned before, the role of the LHb and its projections in these psychiatric disorders will be discussed in more detail in chapter 4.

In summary, both dopaminergic and serotonergic neurons play a major role in the aspects of reward processing, although in a distinct manner. Whereas dopamine neurons provide phasic signals related to reward prediction error, DRN neurons provide tonic signals related to expected and received reward values. In both systems, the LHb has an influence, via its projections through the FR, on these reward processes. The LHb can either inhibit dopamine neurons in the VTA through GABAergic interneurons, or inhibit serotonergic neurons in the DRN. In both systems, dysfunctions can lead to symptoms of psychiatric disorders.

3.4 Cognitive functions of the lateral habenula

In addition to its function in motivational and motor control of behavior, a role for the LHb in cognition has recently been emphasized, particular in relation to spatial learning and attention. Therefore, the last paragraphs of this chapter will briefly discuss the function of the LHb in spatial learning, memory, and attention.

Spatial learning and memory

The involvement of the lateral habenula in spatial learning and memory is proposed by studies in which levels of brain metabolic activity in the habenula (readout for neuronal activity) have been associated with the performance in memory tasks and studies in which the effect of habenular lesions on memory is investigated. In addition, fMRI studies have been conducted on human subjects, normally and cognitively impaired groups, while performing cognitive tasks.

In rats, studies of brain metabolic activity revealed a selective activation of the LHb after the retrieval phase of an odor discrimination task for food reward (Tronel and Sara, 2002). In another study, using the Morris water maze, levels of brain metabolic activity have been investigated in young and old rats. This Morris water maze is a classical test in which the animal (in most studies rats) has to swim in a water maze searching for the (hidden) platform, a well-established test for spatial memory involving the hippocampus (Morris *et al.*, 1982). The animal prefers to be on the platform instead of the water. Villarreal *et al.* (2002) found in their study that after training in the Morris water maze, old and impaired rats showed a reduced metabolic activity in the lateral habenula compared to younger trained rats. The LHb was one of the only few brain areas that showed a reduction in metabolic activity. Since there were no changes in metabolic activity in non-trained young or old rats, the differences found in metabolic activity in trained rats occurred as a result of training-related processes and not from swimming effects in older rats (Villarreal *et al.*, 2002). Supporting evidence for these findings came two years later when Lecourtier *et al.* (2004) performed likewise experiments using habenula-lesioned rats. Concluding from these experiments, several deficits were observed in the habenula-lesioned rats. There were deficits of latency to find the hidden platform and distance swum before finding the platform. Since there were no impairments for the habenula-lesioned animals to find a visual platform in the water maze, the animals must have the necessary motivation and motor abilities to perform the task. Thus, when the

animals are able to see the platform, they are perfectly capable to swim to and climb on this platform, even when their habenula is damaged. These results strongly suggest that their impairment in the hidden platform task is attributed to a deficit of spatial memory, and not to motivation or motor abilities. Furthermore, their impairment cannot be attributed to a small incidental lesion to the hippocampus, as the hippocampus-lesioned group showed no impairments to these tests (Lecourtier *et al.*, 2004). Finally, habenula lesions induced a hypersensitivity to stress, as the learning deficits in habenula-lesioned animals are intensified in stressful situations (Thornton and Davies, 1991). These findings, in combination with the results of Villarreal *et al.*, (2002) strongly indicate that an intact habenula complex is necessary for optimal spatial reference memory.

A number of possible mechanisms could account for the memory impairment observed in the above mentioned studies. These are mechanisms by which habenula damage could result in dysfunction of the hippocampus, a structure that is very important in spatial learning (Squire, 2004; Sargolini *et al.*, 2003; Setlow and McGaugh, 1998).

One of the possibilities is that the involvement of the habenular complex in learning and memory is strengthen by the fact that its lesion alters plasticity in the hippocampus-nucleus accumbens pathway, an important pathway connecting two structures which are both important in learning and memory processes. This phenomenon is likely to be the consequence of an enhanced dopamine release as a result of the habenular lesions (Lecourtier *et al.*, 2006). Not only the hippocampus is a well known structure involved in spatial memory, also the nucleus accumbens is shown to be involved. Using the Morris water maze, impaired spatial memory is shown in mice by dopamine or *N*-Methyl-D-aspartic acid (NMDA)-antagonists injected into the nucleus accumbens (Sargolini *et al.*, 2003). Moreover, lesions of the nucleus accumbens induce memory deficits during the learning phase of the Morris water task (Pennartz *et al.*, 1994).

Another possibility is through the habenulo-raphe pathway, which is an inhibitory influence on the serotonergic cells of the dorsal raphe. The LHb alters serotonergic activity in many structures including the substantia nigra and hippocampus. Lecourtier *et al.* (2004) discusses that induction of long-term depression (LTD) in the hippocampus has been shown to be prevented by serotonin. Since activity of the lateral habenula inhibits the activity of dorsal raphe neurons, it would be possible that in habenula-lesioned animals the dorsal raphe is never properly inhibited, such that LTD in the hippocampus is impaired (Lecourtier *et al.*, 2004). However, the prevention of LTD in the hippocampus by the presence of serotonin has never been confirmed in the literature.

Another possible mechanism in which the habenular complex influences memory impairment also includes serotonin. The LHb participates in the regulation of the medial septum-hippocampus pathway, which is involved in spatial reference memory. As described above, the LHb modulates the activity of raphe serotonin neurons and serotonin transmission. Serotonin, in turn, regulates the neuronal activity of the medial septum and, consequently, of the medial septum-hippocampus pathway. Since these are both important structures for spatial memory, habenular damage could result in memory impairment by influencing this pathway (reviewed by Hikosaka *et al.*, 2008).

Attention

Thornton and Evans suggested already in 1982 that lesions of the habenular complex lead to deficits of attention. Though, this had not been investigated with a well-accepted attention test till 2005. Then, the effects of habenular lesions on attention were explored using the 5-Choice Serial Reaction Time Task (5-CSRTT). The major behavioral consequences of these lesions on the test outcomes were a marked increase in the number of premature responses, which may reflect an impulsive mode of behavior, and alterations in accuracy. Interestingly,

the increase in premature responses occurred direct after the lesions, while the accuracy was not altered in the first tests but became progressively worse as testing progressed. These opposite time-courses indicate that different mechanisms are responsible for the different impairments. This view was supported by the fact that the increase in premature responding was blocked by haloperidol (dopamine antagonist), suggesting that these outcomes are caused by an increased dopaminergic transmission. The decline in choice accuracy was not caused by alterations in dopaminergic transmissions, since haloperidol seemed to have no effect on the choice accuracy. In addition, induction of the dopamine D_2 antagonist decreased premature responding without affecting choice accuracy (Lecourtier and Kelly, 2005, 2007). Considering that the habenular complex plays a role in controlling dopaminergic transmission towards forebrain limbic and striatal structures (as discussed above; Christoph *et al.*, 1986) and given that intra-accumbens infusions of D-amphetamine (increases dopamine release) results in an increase in impulsive, premature responses, with no obvious change in response accuracy during the 5-CSRTT (Robbins, 2002), one could suggest that the increased dopamine release after lesions in the habenular complex leads to an increased amount of premature responses (Lecourtier and Kelly, 2005, 2007).
The observation that choice accuracy is not immediately impaired, but shows a progressive decline is an unusual feature. The exact explanation for this feature is still unclear, but interestingly, progressive alterations in serotonergic and GABAergic function in the interpeduncular nucleus are observed after lesions in the fasciculus retroflexus (Takashita *et al.*, 1990). Therefore, the decrease in choice accuracy could be partially due to the alterations in serotonergic and GABAergic functions in the IPN.

In summary, several lines of evidence suggest an important role of the habenular complex in cognitive functions, such as learning, memory, and attention. Most probably by its direct influence, via projections through the FR, upon serotonergic and dopaminergic transmission towards various key structures involved in cognitive processes, such as the hippocampus and nucleus accumbens.

Chapter 4: Dysfunctions of the habenula in psychiatric disorders

As discussed in previous chapters, the habenular nucleus complex is an evolutionary conserved link between forebrain and midbrain areas. These midbrain areas, such as the raphe nuclei, substantia nigra, and VTA, project widely to most brain areas (Sutherland, 1982). Given the reciprocal connections between the LHb, dopamine and serotonin neurons, it is not surprising that these pathways have been linked to neuropsychiatric disorders in which monoamine-containing neurons have been implicated (Hikosaka *et al.*, 2008). Thus, the DDC circuitry is implicated in various psychological conditions including depression, schizophrenia, and neuropathological responses to addictive drugs (reviewed by Bianco and Wilson, 2009).
This chapter will discuss the link of the habenular complex and the fasciculus retroflexus to these neuropsychiatric conditions. Furthermore, it will elaborate on how the dopamine and serotonin systems are implicated in the pathogenesis of depression and schizophrenia.

4.1 Depression

Many studies have been conducted to one of the major psychiatric disorders, namely depression. The LHb consistently exhibits increased metabolic activity in various animal models for depression, whereas lesions of the habenula improve the behavioral responses of depressed animals. Consequently, both the serotonin and the dopamine system have been associated with depression. In the next paragraph, several crucial findings regarding the LHb and depression will be discussed.

Using three different animal models of depression, Caldecott-Hazard showed in 1988 a specifically increased metabolic activity in the LHb. Moreover, this elevated rate of metabolic activity in the LHb, as well as the depressive behaviors, were prevented by administration of tranylcypromine, an antidepressant drug (Caldecott-Hazard *et al.*, 1988). Earlier studies showed that the habenular complex provides the main forebrain projection to the raphe nucleus and that serotonin neurons in the dorsal raphe were inhibited by habenular stimulation (Wang and Aghajanian, 1977). Since an elevated level of metabolic activity in the habenula contributes to depressive behaviors (above) and the LHb controls the serotonergic neurons, this contribution could occur via LHb's projections through the FR to the serotonergic neurons in the DRN.
In support of this notion, Morris *et al.* (1999) demonstrated that human patients experiencing transient depressive episodes triggered by a reduction in dietary tryptophan (the precursor to serotonin) showed increased regional blood flow in the habenula. This increased blood flow correlates directly with ratings of depressed mood and inversely with plasma tryptophan levels (Morris *et al.*, 1999).
Recently, it was shown that in two different animal models for depression the serotonin levels in the DRN were lower than that in normal animals. Lesions in the LHb improved the behavioral responses of these depressed animals in different tasks. Furthermore, an increased serotonin level was observed in the lesioned-groups of the depressed animals compared with the control animals. Therefore, overactivity of the LHb, and, consequently, depletion of the serotonin levels by inhibiting the raphe may contribute to the pathogenesis of depression (Yang *et al.*, 2008).
As discussed in chapter 3, the serotonin neurons of the DRN play a pivotal role in reward processing. Anhedonia, the failure to experience rewarding stimuli, is one important characteristic of many psychiatric disorders, including depression and schizophrenia. This

feature could be partially due to the above described findings in the animal models for depression.

As discussed in chapter 3, also the dopamine system is crucial in reward-related behavior. The increased metabolic activity in the animal models for depression does not only inhibit the serotonergic neurons in the DRN, but also the midbrain dopaminergic neurons. Overactivity of the LHb would result in more inhibition of the dopaminergic system. This could possibly result in a decreased drive to seek reward, and, consequently, mooddisorders, which may contribute to depressed behavior (Geisler and Trimble, 2008).

4.2 Schizophrenia

Recently, a number of findings suggested that pathology of the habenula and the fasciculus retroflexus could be involved in some of the symptoms of schizophrenia. Although the cause and the effects of the observed habenular dysfunction are unknown, there have been studies regarding potential pathological mechanisms. This paragraph will highlight the most important research conducted upon this topic.

Firstly, in 1992, Sandyk demonstrated that in 87% of schizophrenia patients, habenular calcification has been observed. Since habenular calcification in a normal population is 15%, these findings demonstrate an almost six-fold higher prevalence of habenular calcification compared to normal controls. Although the implications of habenular calcifications for the functions of the structure remain largely unclear, this high rate of calcification patients may play an important role in the pathophysiology of schizophrenia (Sandyk, 1992).
Secondly, the influenza A virus selectively attacks the habenula, paraventricular thalamic nuclei, and brainstem monoaminergic areas (VTA and raphe nucleus), when introduced into the mouse brain via the olfactory bulb. Furthermore, the paraventricular thalamic nuclei can be selectively activated by antipsychotic drugs, indicating that those may represent a target of action for these substances. Since the brain structures targeted by the influenza virus are implicated in the pathogenesis of some neurospychiatic disorders, there might be a potential role for this virus to cause such psychiatric dysfunctions (Mori *et al.*, 1999). In humans, prenatal exposure to influenza A increases the risk to develop schizophrenia (Venables *et al.*, 2007).
Next, research has revealed that the DDC circuitry is particularly susceptible to the neurotoxic effects of addictive drugs. Continuous administration of amphetamine and cocaine has similar neurotoxic effects in the LHb and FR. Most neurotoxic effects of drugs of abuse are due to pharmacologically local neurotoxic results of the drugs on neuronal elements. However, the effects of amphetamine or cocaine on the FR are much more severe; they cause degeneration of axons in the sheath of the FR deriving from the LHb. Interestingly, continuous administration of nicotine causes a specific degeneration of axons from the MHb neurons, which descend in the core of the FR. As discussed in chapter 3, lesions of the habenula and/or FR lead to a higher firing rate of the dopamine neurons in the midbrain. In case of degeneration of the FR, the inhibitory feedback control over dopamine release in the midbrain would drop, resulting in a hyperdopaminergic state (Ellison, 1994, 2002). Several studies have shown that psychosis can be induced primarily by a hyperstimulation of dopamine receptors (reviewed by Ellison, 1994). FR pathology may therefore be involved in various psychoses.

Based on these findings, several mechanisms of how habenula damage could result in symptoms of schizophrenia have been proposed. If damage of the habenula is indeed

involved in schizophrenia, then lesions of the habenula would result in schizophrenia-like symptoms. This hypothesis was investigated by Lecourtier *et al.* (2004); they made lesions in the habenula of experimental animals and tested these animals for several behaviors. The behaviors examined were social interaction, prepulse inhibition (PPI), and memory function, since all these behaviors are proved to be disturbed in schizophrenic patients. A clear impairment was only observed in the Morris water maze test of spatial memory; there were no effects of the habenula lesions on social exploration time or prepulse inhibition. These findings strongly suggest a role for the habenula in spatial memory, but do not support the major role of habenula pathology in reductions of social interaction and PPI in schizophrenia (Lecourtier *et al.*, 2004). Although, It should be taken into account that in another form of social behavior, namely maternal behavior, the habenula plays a pivotal role. This form of social behavior has been proven to be disturbed in schizophrenia (Goodman, 1987; Corodimas *et al.*, 1993).

As discussed in chapter 3, one study has shown that neuronal activity in the LHb is briefly increased in primates experiencing the loss of an anticipated reward. These changes are opposite from those observed in the dopamine neurons. Therefore, this study provides support for the role of the LHb in the generation of a teaching signal in order to select an action (Matsumoto and Hikosaka, 2007). Many patients with psychiatric disorders are not able to use this negative feedback to guide action selection. In support of this notion, in a human fMRI study, subjects (control volunteers and schizophrenic patients) had to perform a difficult mental task and therefore made numerous errors. In error trails where informative (negative) feedback was provided, activation occurred in the habenular complex and the midbrain of the control group. In contrast, the schizophrenic patients exhibited no activation in these areas. These results indicate that impaired activity in the habenula-midbrain projections is correlated with impaired cognitive performance in schizophrenia. The investigators of this study suggested that LHb dysfunction would limit a person's ability to learn from errors, which is one of the most characteristic features of schizophrenia (Shepard *et al.* 2006). These differences may partially account for the maladaptive behaviors of schizophrenic patients.

Another frequent neurocognitive abnormality observed in schizophrenic patients is disrupted attention. As already discussed in chapter 3, some aspects of attention can be influenced by the dopaminergic system. Habenula lesions in rats caused multiple deficits in an attention test that was developed after a performance task of attention, which was used to test schizophrenia patients. These patients appeared to be impaired in this test. Thus, habenula lesions contribute to cognitive impairments observed in schizophrenic patients (Lecourtier and Kelly, 2005). Whether damage to the habenula causes schizophrenia or only contributes to the pathology of the disorder needs further investigation.

The habenular complex is a point of convergence for neuronal information from the basal ganglia and the limbic forebrain. It conveys this neuronal information to monoaminergic mid- and hindbrain areas via a prominent fiber tract; the fasciculus retroflexus (Sutherland, 1982). The lateral habenula mostly innervates the serotonergic neurons in the raphe nucleus and the dopaminergic neurons in the VTA and substantia nigra pars compacta (Figure 9). Through these innervations, the LHb can control the activity of the dopamine and serotonin neurons, and thereby, the levels of these monoamines in the midbrain (Hikosaka *et al.*, 2008).

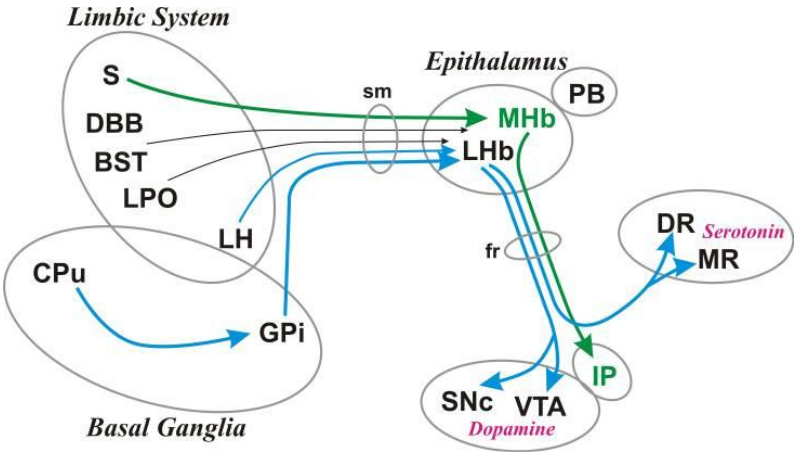

Figure 9: Most important afferent and efferent connections of the habenula. Green and blue lines indicate the axonal connections associated with the MHb and LHb, respectively, black lines are associated with both. The thickness of the line implies the strength of the connection. For abbreviations: see abbreviation list. Figure adapted from Hikosaka *et al.* (2008).

Studies have revealed that lesions of the lateral habenula or the FR result in an increased dopamine release in the VTA, nucleus accumbens, frontal cortex, and striatum. Furthermore, stimulation of the LHb neurons results in inhibition of the dopamine neurons in the VTA and nucleus accumbens (Ji and Shepard, 2007). This suggests that the LHb has an inhibitory influence on the midbrain dopamine neurons. Moreover, stimulation of the LHb neurons inhibits dorsal raphe cell firing (Nakamura *et al.*, 2008). Therefore, it also exerts an inhibitory influence on the midbrain serotonin neurons.

Several pharmacological and behavioral studies have suggested that both the dopamine and serotonin systems are important elements in the brain reward circuitry (Wise, 2004; Schultz, 2006). Since the LHb is implicated in these monoamine systems, it is thought that the LHb also plays a role in reward-related behavior (Shepard *et al.*, 2006). The projections of the LHb towards the midbrain dopaminergic neurons contribute to determining errors in reward prediction. Furthermore, the projections towards the serotonergic neurons of the DRN contribute to the prediction of large rewards. The LHb influences the various reward processes via these distinct manners (Matsumoto and Hikosaka, 2007; Shepard *et al.*, 2006).

Dysfunctions in these systems are believed to contribute in the pathogenesis of several neurospychiatric disorders, for example, depression. Several animal models for depression showed an increased metabolic activity in the LHb, which led to a depletion of serotonin and dopamine in the midbrain. The decreased levels of these monoamines will result in several implications in the reward system: a disability to learn from errors, as well as anhedonia, the failure to experience rewarding stimuli (Yang *et al.*, 2008).

In addition to its implications in depression, the LHb is thought to contribute to the pathology of other neuropsychiatric disorders, such as schizophrenia. Many studies have suggested a

role for the habenula in the pathology of schizophrenia, but the exact mechanism of how habenular dysfunction will contribute to the development of schizophrenia remains unclear. Known is that schizophrenic patients have a higher percentage of calcification of the habenula (Sandyk, 1992). Furthermore, the influenza A virus is thought to attack the habenula en causes schizophrenia-like symptoms (Mori *et al.*, 1999). Several drugs of abuse can also damage the habenula or FR. This leads to a hyperdopaminergic system, as a result of the lack of a higher control circuitry. It is thought that this hyperdopaminergic state of the brain contributes to the pathology of schizopohrenia (Ellison, 1994, 2002). What remains uncertain is if these alterations, for instance the high level of calcification, are the initial changes or if they are consequential to the disorder. Besides, how habenular damage contributes to the pathology of schizophrenia is still unclear. Further investigation into these mechanisms is necessary.
Schizophrenic patients show an impaired cognitive performance; they cannot use negative feedback to guide actions and several forms of social behavior and attention have been proven to be disturbed. Some of these schizophrenia-like symptoms have been observed in animal models with lesions of the habenula or FR. As a result of habenula impairments, cognitive behaviors such as spatial learning, memory, and attention appear to be altered (reviewed by Lecourtier and Kelly, 2007). However, there were also habenula-lesioned animals with normal social behavior, this indicates that not all the behaviors are impaired as a result of the habenular lesions (Lecourtier *et al.*, 2004). It could be possible that the habenula only contributes partially to the pathology of this disorder. Whether habenular damage causes schizophrenia or only contributes partially to the pathology of this disorder needs further investigation.

Only little is known about the development of the habenula and the fasciculus retroflexus. The habenula is a very intriguing and complex structure: it comprises of at least 15 subnuclei and it is implicated in many functions and behaviors (as discussed above). To completely understand how the habenula exerts influence upon these many different brain structures and behaviors, it is important to know what kind of connections they make and how these connections were realized.
A few important axon guidance cues have been discovered to be crucial for a proper development of the habenula and its projections to the ventral midbrain (Funato *et al.*, 2000; Kantor *et al.*, 2004). Still, there are many remaining questions regarding this development that need to be answered. It has been shown that the axons from the MHb comprise the core of the FR, while the axons from the LHb form the sheath of the FR (Kim *et al.*, 2005). How do these differences in core/sheath arrangement develop? Are the axons from the core of the FR directed towards different targets than the axons from the sheath of the FR? How are these differences in target directions accomplished? Are there more axon guidance cues that play a role here? More research into axon guidance cues at the crucial age of E12-E16 is necessary to answer these questions.
In a recent study by Dugan *et al.* (2011) Slit/Robo signaling molecules showed to play an important role in maintaining proper dorsoventral positioning, promoting straight axonal growth through the diencephalon, and preventing ectopic projections into the ventral midline. These results were shown to be crucial for a proper trajectory of the ventral mesodiencephalic dopaminergic (mdDA) axons (Dugan *et al.* 2011). One could assume that these signaling molecules would also prevent the FR from ectopic projections into the floor plate. Therefore, more investigation on these signaling molecules in relation to the FR is needed.

Future perspectives

Considering the anatomical and neurochemical complexity of the habenular complex, much work remains to reveal the details on how this complex exerts its multiple effects on behavior and to establish if and how its pathology contributes to neuropsychiatric disorders, such as depression and schizophrenia. As described in this paper, the habenula is involved in many functions, from which only a few were discussed here.

Lesions of the habenula or the FR have an impact on both the dopamine and serotonin systems. Some of the targets of the VTA and DRN towards structures as the prefrontal and cingulate cortices need to be explored, using tracing studies, as these interactions may contribute to the cognitive deficits. The hypothesis that habenula dysfunction contributes to the cognitive impairment in depressed- and schizophrenia patients deserves further consideration. Moreover, since the habenula exists of at least 15 different subnuclei (Andres *et al.*, 1999; Geisler *et al.*, 2003), damage of only a few subnuclei would not necessary result in deficits of all the behaviors that the habenula is involved in. Therefore, it would be interesting to research the different functions of the many subnuclei. Which subnuclei project to what midbrain area? This could be done by combining studies of specific lesions and localized stimulations. Then depending on the exact distribution of the damage, multiple mechanisms may contribute to the behavioral disturbances.

It should also be interesting to conduct more research to habenular dysfunction at cellular levels. In order to understand the substrates for lesion-induced effects on, e.g. learning and memory, synaptic plasticity could be measured in the prefrontal cortex or hippocampus. Are there differences in neuronal strength of the neurons projecting to these areas, before and after habenula lesions? What are the exact influences of habenula lesions on these brain areas that are important for learning and memory? Furthermore, the receptors in these areas could be investigated. Are there different types of receptors in the target areas of these monoamines? What are the results of habenula lesions on these receptors? Besides, the differences between immediate and progressive lesion-induced effects could be measured at cellular level.

Finally, more research should be done to the development of the habenula and the fasciculus retroflexus. In order to completely understand the functions of the habenula and its projections in the many different behaviors, it is important to know how these connections were realized. The habenula projects to many different brain areas. Little is known about the development of the FR, but it remains unclear how the axons reach and innervate the different destinations. More research into this developmental process in necessary in order to understand how these projections were realized.

Dugan *et al*. (2011) briefly discussed the importance of axon guidance research. Since stem cell research is making major progress, in neurodegenerative diseases, transplant therapies are being considered. The difficulty of these therapies is to let the neurons grow along appropriate pathways to find their targets. Understanding of how (dopaminergic) neurons grow and navigate through their environment is necessary to increase the efficiency and success of these transplants. More research to different signaling cues could provide information necessary for clinical therapy.

This paper has focused on depression and schizophrenia, it proposed that damage to the habenula or FR could contribute to the pathology of these neuropsychiatric disorders. It would be very interesting to investigate if and how the damaged axons of the FR could be replaced with transplant therapies. Then, it would be necessary to know exactly which axon guidance molecules are essential to guide the transplanted axons along their appropriate pathways towards their targets.

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