Aus dem Centrum für Anatomie
der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

DISSERTATION

Genomic-morphological analysis of the rat and mouse habenulae uncovers a high molecular heterogeneity and indicates involvement in the regulation of feeding and energy balance

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Franziska Wagner

aus Suhl

Datum der Promotion: 10.03.2017

Contents

1. Summary

1.1. **Abstract (english)** 1

1.2. **Abstrakt (Deutsch)** 2

1.3. **Introduction**

- 1.3.1. How to decide? 3
- 1.3.2. The medial and lateral habenula are important relay stations 3
- 1.3.3. Biological functions of the medial and lateral habenular complexes 5
- 1.3.4. Aim of this investigation 5

1.4. **Methods**

- 1.4.1. Chemicals 6
- 1.4.2. Animals 6
- 1.4.3. Analysis of data derived from the Allen Brain Atlas 6
- 1.4.4. Morphological and immunocytochemical analysis 6
- 1.4.5. In-situ-hybridization 7
- 1.4.6. Generation of Riboprobes 7
- 1.4.7. Microarray analysis 8

1.5. **Results**

- 1.5.1. Morphological and cythochemical comparison of rat and mice habenula 8
- 1.5.2. Analysis of gene expression patterns of habenular subnuclei using the Allen Brain Atlas 9
- 1.5.3. Microarray expression analysis of rat habenula 10
  - 1.5.3.1. Genes with enriched expression in the MHb 10
  - 1.5.3.2. Genes with enriched expression in the LHb 11
  - 1.5.3.3. Genes with enriched expression either in the MHb or in the LHb as compared to each other 11
- 1.5.4. Morphological evidence for major transmitter systems in the MHb and LHb
  - 1.5.4.1. Cholinergic system 11
  - 1.5.4.2. Glutamatergic system 11
  - 1.5.4.3. GABAergic system 12
  - 1.5.4.4. Dopaminergic system 12
  - 1.5.4.5. Serotinergic system 12

1.5.4.6. Peptide transmitter systems 13

1.6. Discussion

1.6.1. Characterization of the mouse habenula 13

1.6.2. Methodical limitations of the Allen Brain Atlas and microarray analysis 13

1.6.3. Potential roles of habenular subnuclei in diverse biological function 14

1.6.4. Interpretation of potential markers for habenular subareas 15

1.6.5. Involvement of the habenula in homeostatic behaviors 15

1.6.6. Potential involvement of the habenula in autism 16

1.6.6. Concluding remarks 16

1.7. References 17

2. Anteilserklärung / eidesstattliche Versicherung 21

3. Published Manuscripts 23

3.1. „Correlating habenular subnuclei in rat and mouse by using topographic, morphological, and cytochemical criteria” 24

3.2. „Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries” 37

3.2. „Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance” 57

4. Curriculum vitae 84

5. Bibliography 86

6. Acknowledgements 87

1. Summary

1.1. Abstract

Positive as well as negative reward-prediction are elemental for survival and influence our behavioral state. Especially the lateral habenula (LHb) has received much interest because it has been recognized as the potential center of a “negative-reward system”. With its multiple prominent involvements in the regulation of neuromodulators, particularly of the dopamine, serotonin and cholinergic system, a further elucidation of the detailed synaptic connectivity of habenular subnuclei is urgently needed.

The mammalian habenula consists of medial (MHb) and lateral (LHb) nuclear complexes. Considerable knowledge has accumulated especially concerning the subnuclear structure and the connectivity of the habenula and its neurons. In contrast, attempts to find criteria to classify habenular neurons into separate groups with potentially different biological abilities have largely failed, most likely due to the lack of appropriate marker proteins. One important tool to approach this dilemma is available in form of the Allen Brain Atlas, which provides detailed expression patterns of about 21,000 transcripts. These data, however, are valid in mouse only. Consequently, the mouse habenula was characterized (see [1]), so it was possible to use the Allen Brain Atlas for the investigation of enriched transcripts in the mouse habenula (see [2]). Unfortunately, this approach failed to detect new marker genes of the habenula.

Thus, in a microrarray study (see [3]) MHb and LHb or selected subnuclei were analysed for characteristic transcripts or proteins, which may shed light on the detailed biological functions of these areas. Quite surprisingly, our data indicate potentially inhibitory effects of acetylcholine and glutamate in the habenula. In addition, the partly absence of the K-Cl cotransporter 2 (KCC2) supports a largely excitatory role of GABAergic transmission especially in the MHb. Furthermore, several G-protein related receptors (Gpr83, Gpr139, Gpr149, Gpr151, Gpr158) and many feeding-related neuropeptides are prominently expressed in the habenular region, indicating that its involvement in the regulation of food consumption and energy expenditure may have been underestimated so far.

1.2. Abstrakt

Belohnung einer adäquaten Reaktion, und das Ausbleiben einer Belohnung bei einer inadäquaten Reaktion sind notwendige Mechanismen, die das Überleben sichern. Als potentielles Zentrum des „Negative-Reward-Systems" steht eine Hirnregion, die laterale Habenula, im besonderen Fokus neurowissenschaftlicher Forschung. Die Habenula setzt sich aus einem medialen (MHb) und einem lateralen Unterkernkomplex (LHb) zusammen. Die vielseitige Vernetzung dieser Unterkerne im Zusammenspiel verschiedener Transmittersysteme macht eine differenzierte Betrachtung der Habenula notwendig.

Für die Habenula der Ratte gibt es detaillierte Betrachtungen zur Unterkernstruktur und Morphologie der Neurone. Versuche, den Neuronengruppen verschiedene spezifische biologische Funktionen zuzuschreiben, sind bisher gescheitert. Insbesondere molekulare Markerproteine könnten hier die weitere Klassifizierung erleichtern.

Mit dem „Allen Brain Atlas" steht jetzt eine open-access Datenbank zur Verfügung die über 21.000 Transkripte auf Maushirnschnitten charakterisiert. Durch die morphologische Charakterisierung der Habenula der Maus (vgl. [1]) konnten wir diese Daten erstmals auch für die Unterkerne der Habenula nutzen (vgl. [2]). Zusätzlich konnten einzelne, bisher nicht bekannte Unterkerne identifiziert werden (vgl. [1]). Auffällig war eine sehr heterogene Zusammensetzung der einzelnen Unterkerne (vgl. [2]), hinweisend für eine komplexe Zusammensetzung der einzelnen Kerngruppen. Die Untersuchung zeigte auch, dass die Expression des GPR151-Proteins, welches bereits als Marker für die Habenula genutzt wird, nicht selektiv für Neurone dieser Region ist (vgl. [2]).

In einer Microarray-Studie erfolgte in einem weiteren Schritt die Untersuchung des Expressionsprofils der LHb und MHb der Ratte (vgl. [3]). Hier zeigte sich ebenfalls ein heterogenes Muster der Neuronentypen. Interessant zeigte sich die Verteilung des K-Cl Kotransporters 2 (KCC2), welcher in fast allen Neuronen des adulten ZNS vorkommt und K+ mit Cl- aus Neuronen transportiert. In den meisten Neuronen des adulten ZNS ist Cl_i daher negativer als das Ruhemembranpotential. Dies ist die Voraussetzung für die Entstehung von hyperpolarisierenden Chlorid-Ionenströmen. In der MHb fanden sich Neuronengruppen ohne KCC2, als möglichen Hinweis für eine regional exzitatorische Wirkung GABAerger Projektionen. Des Weiteren zeigte sich eine prominente Expression verschiedener G-Protein gekoppelter Rezeptoren (Gpr83, Gpr139, Gpr149, Gpr151, Gpr158) und Nahrungsaufnahme-assoziierter Neuropeptide in der Habenula als Hinweis für eine wesentliche Bedeutung der Habenula für Homöostase, Nahrungsaufnahme und Energiestoffwechsel (vgl. [3]).

1.3. INTRODUCTION

1.3.1. How to decide?

A major question among neuroscientist is to understand the “Where?” and the ”How?” we decide what we “want”. A key motivation here is reward. The pleasures of food intake, when we are hungry, of drinking when we are thirsty, and of sexual behavior are the elemental impulses keeping populations alive [4]. The experience of pleasure as a very strong drive in human acting has been recognized since greek philosophers (Epicurism). About 2300 years later, Olds and Millner report evidence “indicating various reward places” within the animal brain [5]. Misfunction of this reward system results in addiction, depression, anhedonia, or autism [6, 7]. Expanding this idea to other actions like social contacts opens reward as eventually the main drive in the intensely discussed “free will” [8].

Apparently “wanting” and “liking” are psychologically and neurobiologically different components of reward processing (incentive sensitization hypothesis, [9]). “Wanting” classically is described as “incentive salience to reward related stimuli” and mainly is processed via dopaminergic pathways [10]. In contrast, “liking” is defined as hedonism [9]. Hedonism by its own “occurs, when a satiated individual consumes food mainly because of its palatability” [11].

The release of dopamine depends on the evaluation of reward predictions [12]. On the contrary, the absence of an expected reward or the prediction of a negative outcome, results in “negative reward” (negative prediction error; [12]). Neurochemically “dopaminergic neurons in the ventral tegmental area (VTA) are depressed” [4] in negative reward situations. Consequently, tracing and lesions-experiments aimed to understand, which neuroanatomical structures are involved in negative reward processing [13].

Stimulating the lateral habenula (LHb) inhibits about 90% of all VTA neurons [13, 14], whereas on the opposite, lesioning the habenula increases dopamine metabolism [15]. These experiments suggest a reward and also negative reward based modulation of VTA-dopaminergic neurons by habenular input [16].

1.3.2. The medial and lateral habenula are important relay stations

Since 1920 investigators aimed to understand the habenular complex and its diverse connections [17, 18]. The mammalian habenula consists of two separate areas, the lateral (LHb) and medial (MHb) habenular complex. Morphology, connections (Fig. 1) and biological functions of both complexes are largely different.

The LHb receives afferents mostly via the stria medullaris (Fig. 1). Incoming fibers, excitatory as well as inhibitory, mainly are derived from the ventral striatum, and the preoptic area and lateral hypothalamus [19]. Surprisingly also an excitatory projection from the entopeduncular nucleus (EP; the rodent homologue to the GPi in primates) has been recently identified [20]. This brain region, previously thought to be GABAergic only, has been identified to have a “predominately excitatory glutamatergic projection” to the LHb [20].
The medial habenula (MHb) mainly receives GABAergic afferents from the medial septum and the diagonal band of Broca (DB). Monaminergic afferents stem from the mesencephalic dorsal and median raphe, the triangular septal nucleus (TS; glutamatergic), the locus coerulus (LC; noradrenergic) or from the ventral tegmental are (VTA; dopaminergic) [19, 21, 22, 23].
The majority of efferent axons of the habenula form the fasciculus retroflexus (FR, Fig. 1).

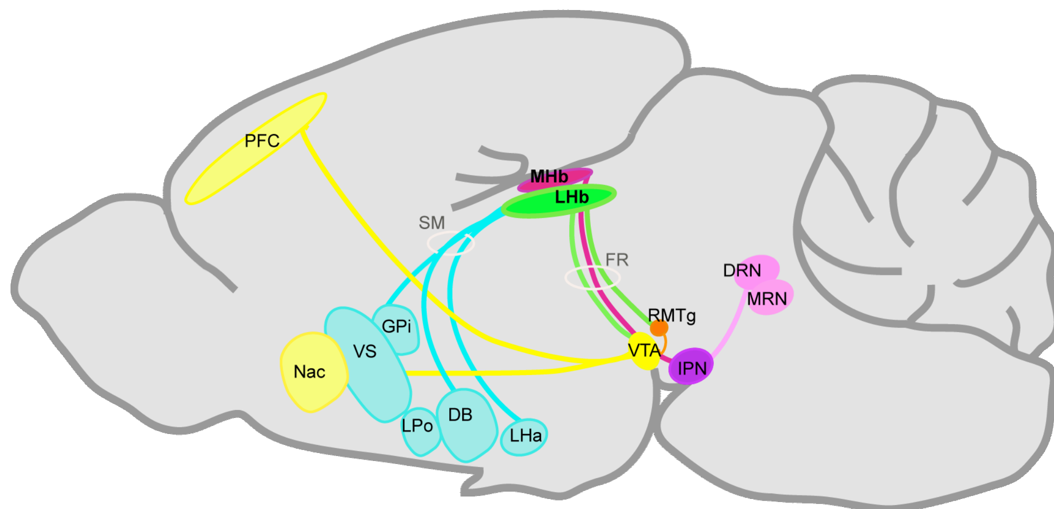

Fig. 1: Schematic representation of main connections of the rat habenula
Afferents (blue) to the habenula via the stria medullaris (SM) are mainly from the limbic system. The MHb sends outputs (pink) mainly to the interpeduncular nucleus (IPN), which projects to the raphe nuclei. The LHb sends efferents (green) mainly to the ventral tegmental area (VTA), also indirect via the rostromedial tegemental nucleus (RMTg). Several other connections are not shown. *Abbreviations:* VS - ventral striatum; FR - efferents via the fasciculus retroflexus; DB - diagonal band of Broca; GPi - globus pallidus internus; LPO -lateral preoptic area; PFC - prefrontal cortex; LHa - lateral hypothalamic area, DRN - dorsal raphe nucleus, MRN - medial raphe nucleus

The predominant output of the LHb is glutamatergic [24, 25] and, directly or indirectly, projects to the VTA, bilaterally to the median and dorsal raphe nuclei [26, 27], and to the cholinergic pedunculopontine and laterodorsal tegmental nuclei (PPTg and LDTg; [28]). Terminals of LHb neurons in the VTA are predominantly glutamatergic whereas the VTA mainly (about 60%) contains dopaminergic neurons with intermingled GABAergic (about 30 %) and glutamatergic

neurons [29]. LHb and VTA are connected by a direct pathway and, as recently found, by an indirect pathway via the rostro-medial tegmental nucleus (RMTg) [27, 30]. The inhibitory influence of the LHb on the VTA has been shown to be mediated by the RMTg, their GABAergic neurons receive a dominant input from the LHb [27, 30]. Additionally a recent study describes an additional population of dopaminergic-GABaergic neurons in the VTA that suppresses LHb output [31]. Neurons of the MHb are mainly glutamatergic and cholinergic projecting to the interpeduncular nucleus (IP) [32]. Recent work suggests that the MHb-IP pathway is a part of several circuits regulating addiction and withdrawal and works as a link between the limbic forebrain and the midbrain [33, 34].

1.3.3. Biological functions of the medial and lateral habenular complexes

Despite its small size, the lateral habenula is associated by a broad spectrum of functions and diseases. The LHb is involved in the regulation of sleep, nutrition, circadian rhythm, stress response, or pain processing [35-37]. A recently discovered the lateral habenula is also mainly involved in the processing of negative reward [38].

"Quite recently the biological importance of the MHb was emphasized by the fact that the alpha-3, alpha-5, and beta-4 nicotinic receptors, which are implicated in heavy tobacco use and decreased success in smoking-cessation therapy in humans, are prominently expressed in the MHb and its major efferent target, the interpeduncular nucleus [39]."[1] The MHb also may play an important role in anxiety, fear and primary reinforcement and is involved in the "regulation of the hedonic state" [33, 34, 40, 41].

1.3.4. Aim of this investigation

The involvement of the habenula in negative-reward, or for example pain processing, leads to substantial scientific interest. The divided subnuclear structure suggests also a biological functional separation of habenular subnuclei. The primary aim of the current investigation was to detect novel molecular markers, which should allow to classify habenular neurons in to individual neuron types and to localize such cell types in habenular subareas.

Consequently, we first analyzed and characterized the mouse habenula (see [1]), then used the datasets of the Allen Brain Atlas for the investigation of enriched transcripts in the mouse habenula (see [2]). Unfortunately, this approach did not result in new marker genes of the habenula. In a third investigation (see [3]) a microarray analysis of expressed transcripts in MHb and LHb was proceded, to find transcripts or proteins, which may shed light on the detailed biological functions of these areas.

1.4. METHODS

1.4.1. Chemicals

Chemicals were obtained from Sigma, Taufkirchen, Germany, if not indicated otherwise. Sources of primary antibodies are described below. Biotin labeled secondary antibodies and the Elite AB-complex were from Vector (Vector Laboratories, Burlingame, CA, USA).

1.4.2. Animals

All animal experiments were approved by the Regional Berlin Animals Ethics Committee and conducted in strict accordance with the European Communities Council directive regarding care and use of animals for experimental procedures. Subsequent data are based on the analysis of about 500 sections derived from mice (n=6) and about 800 sections derived from male Wistar rats (n=18) [1; 3].

1.4.3. Analysis of data derived from the Allen Brain Atlas

„The Allen Mouse Brain Atlas (ABA), a genome wide atlas with datasets of the expression of about 21,000 transcripts [46] was used to detect transcripts, which are preferentially localized in the habenular region."[2] This atlas provides different tools to perform virtual experiments based on the datasets (see methods section and Fig. 1 in [2]). The "Anatomic Gene Expression Atlas" (AGEA), a dataset out of 4,082 transcripts in coronal mouse brain sections, offers the possibility to retrieve a set of genes highly expressed in a defined region (seed voxel, for detail see [2] and Fig. 2 A in [2]). Virtual experiments to find transcripts whose expression in the seed voxel is locally enriched in the lateral division of the LHb, the oval subnucleus of the lateral division of the LHb and the parvocellular nucleus of the medial division of the LHb. The "Gene Finder-tool" then retrieves a set of"[2]. The "Correlation-tool" offers the possibility to find transcripts with similar expression to a selected transcript pattern in a selected region of the mouse brain (for detail [2]).

1.4.4. Morphological and immunocytochemical analysis

To evaluate the morphology of the mouse in correlation the rat habenula, coronal sections with Klüver-Barrerea and Nissl-staining and immunhistochemistry were analysed. Primary antibodies (Table 1 in [1]), studied in rat [42] were used for cytochemical analysis. The protocol for staining freely floating for 25 µm cryrostat sections is described in detail in author's manuscripts [1,3], as are the protocol for Nissl and Klüver-Barrea stain [1]. Acetylcholine esterase activity

(AChE) was visualized by a modified Karnowski protocol (for details see [1]).

"Microscopic images were obtained with an upright Leica DMRB light microscope connected to a high-resolution digital camera (Olympus SP-55UZ). Primary photographs were adjusted for brightness, color, and contrast in Adobe Photoshop CS3 (10.0) and arranged using Adobe Illustrator CS3 (13.0.0)."[1, 2, 3]

1.4.5. In-situ-hybridization

Freely-floating in-situ-hybridization has been established in the authors laboratory and is described in detail in authors manuscripts [2, 3]. In short, serial cryrostate brain sections of 25 µm were incubated with selected riboprobes for about 16 h at 56°C. After stringency washing, sections were incubated with a mouse primary antibody against digoxigenine (Dig). After 24 h, a biotinylated secondary antibody was added for 8 h at room temperature. Freely-floating sections were washed and treated with an avidin-biotin-peroxidase-complex. Using the CARD-technique (Catalyzed Reporter Deposition, scheme Fig. 2) the Dig-based signal was amplificated [44, 45].

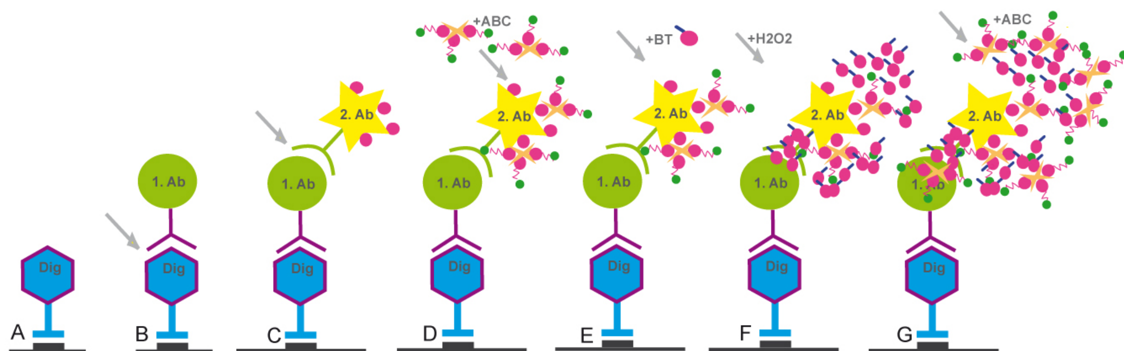

Fig. 2: Schematical representation of CARD-reaction (according to [44, 45])
(A) The Dig-labeled riboprobe (light blue) binds to RNA (grey). (B) The primary antibody binds to the Dig-labeled riboprobe. (C) The secondary biotin-labeled (pink dot) antibody (yellow star) binds to the primary antibody. (D) The avidin-biotin-peroxidase complex (ABC; Avidin: orange cross; peroxidase: green dot) binds to the biotin of the secondary antibody. (E) After incubation with biotinyl-tyramide (BT), the BT is oxidized by the peroxidase forming BT-radicals (see F). (G) The deposits of biotinyl-tyramide radicals close to the antigen again are labeled with ABC. Thus, the increased numbers of peroxidase molecules result in a heavily amplified signal.

1.4.6. Generation of Riboprobes

To obtain highly specific riboprobes, primers (Table 2 in [3]) were designed using http://biotools.umassmed.edu/bioapps/primer3_www.cgi. PCR products were sequenced and cloned into a pGEM-T Vector (Promega). RNA probes were labeled with digoxigenine (Dig-RNA labeling Kit Roche).

1.4.7. Microarray analysis

„For microarray analysis wistar rats (n=5) were perfused for three minutes with ice-cold PBS. For probe generation, nine samples of the habenula, four complete lateral and five complete medial habenula, and three adjacent pieces of thalami were obtained by microscope assisted manual microdissection“. The „Affymetrix GeneChip® 1.0 ST Arrays“ were hybridized with cDNA of brain tissue samples by the "Laboratory for Functional Genome Research", Charité-University Berlin. All measured probe intensities can be found as "supplement table 1" in [3]. Microrarray data were analysed with the aid of RMA (Robust Multi-array Average) algorithm. „Limma“ was used to compare expression between MHb versus thalamus, LHb versus thalamus, and MHb versus LHb [43]. „Limma“ was chosen because it leverages the parallel design of microarrays to better estimate variability for a specific gene in experiments with a small amount of samples. Data were considered to be statistically significant with a false discovery rate (FDR) below 0.05. Transcripts with an intensity value < 6 in at least one of the two nuclei and transcripts with Fold Changes (FC; defined as normalized mean difference between two microarray intensity values) below 1.5, indicating a minimum of 50% difference in expression intensity, were ignored. Original data are available as supplemental tables in figshare (https://figshare.com/s/52cb26422eb2a3f3f822).“[3]

Comparing gene expressions MHb against LHb and thalamus, vice versa was done following criteria: FDR values <0.05, FC values > 1.5. The resulting list was subsequently cleared of transcripts not directly related to the aim of the present investigation (e.g. house-keeping proteins, developmental factors, ion channels and synaptic signaling).

1.5. Results

1.5.1 Morphological and cythochemical comparison of rat and mice habenula

Overall comparison of survey micrographs gives a similar appearance of rat and mouse habenula (Fig. 1; in [1]). In a first approach, the distribution of main cytochemical "markers" [42], such as acetylcholine esterase enzyme activity (AChE), neurofilament- (NF-) or Kir3.2-immunoreactivity (IR), were tested in mouse and compared with data from the rat habenula. For example, the parvocellular subnucleus of the medial division of the LHb (LHbMPc) in rat contains AChE enzyme activity, but is largely devoid of NF-IR and Kir3.2-IR. A similar pattern has now been found for the mouse (Fig. 2 A-D; in [1]). An analoguoes procedure was done for other nuclei of the LHb (Fig. 2- 3; in [1]). The morphology of the rat habenula in Klüver-Barrera technique could be used to identify the five known MHb subnuclei in the mouse (Fig. 4; in [1]).

Combination of morphology knowledge and immunocytochemical location of serotonin (Fig. 4 C; in [1]), Kir 3.2 (Fig. 4 D; in [1]), NF-IR and AChE activity (Fig. 4 B; in [1]), allowed to distinguish three additional subnuclei: the lateral, superior and medial subnucleus of the ventral MHb (MHbVl, MHbVs, MHbVm) in the lower field. In addition two subnuclei in the upper field of the MHb, the dorsal and the superior subnucleus of the dorsal MHb (MHbD, MHbS) were identified. Conspicuously neurons were found in an area lateral to the MHb (Fig. 7; in [2], which we here termed HbX area. "On closer inspection it remains unclear, whether this field should be attributed to the medial or the lateral habenular complex. Analysis at high resolution reveals the idea of this region as composition of intermingled MHb and LHb neurons. The biological meaning of this region remains unclear."[2] Our recent scheme, which provides an overview of the localization of subnuclei in the MHb and LHb (see Fig. 6; in [1]) has been modified here to include the HbX area (Fig. 8; in [2]).

1.5.2. Analysis of gene expression patterns of habenular subnuclei using Allen Brain Atlas

"When the subnuclear composition is well described and the hodological connections of the habenula seem to be relatively unrelated to its subnuclear organization"[2], the complexity of individual cell types within the habenula has to be looked at in detail. An attempt here is to analyse gene expression and classify cell groups and cell-types. The Allen Brain Atlas-database provides the localization of approximately 21,000 transcripts, whereas 4,082 transcripts were given in coronal sections. These served as source for this analysis. To investigate transcripts locally enriched in the habenula, the "AGEA-tool" provides the possibly to select suitable seed voxels (SVs**)**. Datasets for three SVs within the lateral habenula were further analysed (Fig. 2; in [2]). Many transcripts showed the expected localizations, but several "highly" expressed genes were not even found in whole habenular area (e.g. Ramp3, Fig. 2 B; in [2]), or show up with densely packed signals in adjacent regions (e.g. Calb2 and Kcng4, Fig. 2 C, D; in [2]). Apparently the "AGEA-tool" is compromised by the fact of size and orientation of selected seed voxel, which do not precisely correspond to habenular morphology. To overcome this problem, 80 genes per SV were observed by hand with the "high resolution viewer". The "Correlation-dataset" allowed virtual comparison of the distribution of a selected gene to other genes in the database. Additionally the "Correlation-tool" was to used to find genes, similarily distributed like GPR151, a formerly known "marker gene" of the habenula [47, 48] (see Fig. 3 C; in [2]). Manual analysis of now about 500 transcripts revealed in 138 genes, which show a dominant signal within the habenula. To detect potentially enriched gene ontology terms, the resulting gene-list was analysed by "GOrilla analysis". With this limited number of genes, no significantly

enriched pathways were observed (see for detail [2]). Transcript locations were grouped to 10 distribution patterns (Table 1, Table 1 supplemental; in [2]). Only few patterns respect known subnuclear delineations (Table 1, pattern 1-6, Fig. 4 A, B, G, H, I, J; in [2]). “Several distributions could be grouped in similar patterns (Table 1 pattern 7-10; in [2]). In contrast to the situation in LHb, where protein distribution patterns were greatly heterogenous, in the MHb many transcripts tended to respect subnuclear boundaries (Fig. 6; in [2]). In addition, comparing selected distributions, suggests the presence of a novel subfield adjacent to the subregion of the medial subnucleus of the ventral MHb (MHbVm). This region had not been recognized in earlier reports, and is now termed intermediate field of the ventral MHb (MHbIm) (Fig. 8; in [2]). Quite interestingly, Cartpt (Fig. 6 I; in [2]), Sema3d, and some others (Table 2; in [2]) covered the lateral edge of the lateral subnucleus of the ventral MHb (MHbVl) (Fig. 8; in [2]). This narrow field may correspond to an area, that had been termed border zone (BZHb) [19].”[2]

1.5.3. Microarray expression analysis of rat habenula

“Microarray experiments using chips for MHb, LHb and adjacent thalamus with about 27,342 probes resulted in data for about 17,745 RNAs representing protein-coding genes for each region (supplemental Table 1; in [3]). “[3] Aiming to find regionally enriched transcripts, the total number of differentially expressed transcripts, which display elevated expression in the MHb as compared to the thalamus, the LHb and the LHb compared do the thalamus were compared using a Venn diagram (Fig. 1; in [1]). “Interestingly, while differences between the biological functions of the MHb and the LHb are not really obvious, the MHb expression-profile was very different from the thalamus as well as from the LHb, while differences between the thalamus and the LHb were less obvious.”[3] “General analysis of the differential expression in LHb to MHb disclosed very high FDR-values.”[3] This indicates that the LHb and the MHb are genetically much less related as LHb and thalamus.

1.5.3.1. Genes with enriched expression in the MHb

MHb expression values were compared to thalamus data to identify potential transcripts with potentially selective expression in the MHb. Statistical and functional criteria (see methods section) leaved 34 genes for further analysis (Table 3 in [3]). The gene with the highest differential expression in the MHb with respect to the thalamus is the GPR151, a formerly known “marker gene” of the habenula gene (see [2]), underlying correctness of data acquisition. Other highly ranking genes were Tac1, Tac3 and 5HT5b (see [3]).

1.5.3.2. Genes with enriched expression in the LHb

LHb expression values were compared to thalamus data to identify potential transcripts with potentially selective expression in the LHb. Statistical and functional criteria (see methods Following statistical and functional criteria (see methods section) leaved 15 transcripts (Table 5; in [3]) for further analysis. To increase the number of gene the FDR was relaxed to 0.2 (Table 4; in [3]) with leaving about 22 genes. "Again the GPR151 and HTR5b transcripts were top ranked."[3] "Interestingly cerebellin 1 and 2 showed up, which are also found in the ventromedial hypothalamus, an area which is tightly involved in feeding and energy control, indicating a potential role of the LHb in such homeostatic functions."[3]

1.5.3.3. Genes with enriched expression in the MHb or in the LHb as compared to each other

To observe main biological differences between MHb and LHb expression values of MHb and LHb were compared. Following statistical and functional criteria (see methods section) leaves 43 transcripts with MHb > LHb and 35 transcripts with LHb > MHb (Table 5; in [3]) for further analysis. "An interesting example was the KCC2 chloride transporter, which determines whether GABA has an excitatory or an inhibitor function, which is much higher in the LHb as compared to the MHb (see discussion section)."[3]

1.5.4. Morphological evidence for major transmitter systems in the MHb and LHb

1.5.4.1. Cholinergic system

The MHb is relevant in the cholinergic, especially in the nicotinergic withdrawl pathway [39]. In line with this data, expression of the Chrna5-Chrna3-Chrnab4 gene-cluster, known to be related to nicotine withdrawl, is also enriched in our dataset. Surprisingly these subunits are also highly expressed in the LHb. In contrast to the nAChR subunits, the presence of the muscarinic subunits (mAChR) is less documented (Table 5b; in [3]. The subunits mAChR2, 3 and 5 are among the genes with a significant high expression in the LHb as compared to the MHb, The locations of mAChR2 and -3 were verified in mRNA and protein level, confirming our microarray results (Fig. 2; in [3]).

1.5.4.2. Glutamatergic system

"Most neurons in the MHb as well the LHb use glutamate as major transmitter (Omelchenko et al., 2010). In line microarray data show up with high expression values for the vesicular glutamate transporters vGluT1 and vGluT2 (supplemental Table 1; in [3]). Glutamate often is colocalized with other transmitters such as ACh or dopamine and such neurons often use the

vGluT3 transporter [49]. VGluT3 positive elements are found prominent in the LHb and MHb (Fig. 3 A-C; in [3]), in contrast to earlier observation [50].”[3]

1.5.4.3. GABAergic system

“The habenula receives a massive inhibitory input, in contrast to its meager number of internal GABAergic neurons [24, 30]. An increased presence of GABAergic, GAD67-positive neurons in the LHb were found, by using a more sensitive in-situ-hybridization method (CARD-technique) (Fig. 4 B; in [3]). Thus microarray expression data indicating a prominent presence of GABA-A subunits alpha1, alpha3, alpha5, beta2 and beta3 is not surprising. The localizations of alpha1, beta2, beta3, and delta subunits were also verified by immunocytochemistry (Fig. 4 C-F; in [3]). The understanding of the functional diversity of GABAergic receptors was increased by analyzing KCC2-chloride transporter distribution. This transporter defines, whether opening GABA-A receptor results in excitatory or inhibitory effects. Here our microarray data show a higher expression in the LHb than in the MHb (Table 5b; in [3]), which was also regionally analysed by immunohistochemistry (Fig. 4 H; in [3]).”[3]

1.5.4.4. Dopaminergic system

“Dopaminergic input to the habenular area, documented by tyrosine hydroxylase (TH) positive axon terminals, is well known [1, 42, 51]. Microarray data support monaminergic mechanisms in the habenula (high expression values for monoaminoxidase A and B (MAO-A and MAO-B), dopamine receptor 1 or 2 (DRD1 and 2) (Table 5b and 2; in [3]). Surprisingly mRNA for the DRD5 is easily detected in the LHb, while the well described distribution of DRD4 [52] is not expressed significantly in our dataset (supplemental Table 5; in [3]).”[3]

1.5.4.5. Serotinergic system

“Microarray data supporting serotingergic modulation in the habenula (Table 5 in [3]). A serotonergic input [53] is described, and our microarray analyses and riboprobes display enriched signals for 5-HT1B and 5-HT2C receptor expression (Fig. 5 A, C and D; in [3]) especially in the LHb (Table 5; [3]). As 5-HT1B receptors mostly (but not exclusively) are localized presynaptically [54], they may represent the molecular basis for the inhibitory effects of serotonin on incoming axons from the entopeduncular nucleus in the LHb [20, 55]. Unfortunately, due to the lack of specific antibodies, our immunocytochemical attempts to directly visualize the 5-HT1B receptor protein in the habenula were unsuccessful so far.“[3]

1.5.4.6. Peptide transmitter system

"Focusing on a potentially underestimated role of the habenula in controlling energy homeostasis, quite a lot of other transcripts showed up, which are known or are suspected to be involved in the control of feeding or energy expenditure (e.g. Adcap1, Nppa, Sstr2, Sstr4, Mcr3 Brs3, Gpr139, Gpr151; Cbln1, Bblen2, Sik1, Aplnr, Adipor2, Gpr83, Ntsr2, Gpr149, Gpr158; for detail see [3]). So far, several of them like NPY, cholecystokinin could be identified in the habenula by in-situ-hybridization or immunocytochemistry (Fig. 7 E-H; in [3]). Much to our surprise, the transcript with the relative highest expression in the LHb compared to thalamus is neuronatin (Nnat, Table 4; in [3]). This peptide is strongly involved in the regulation of feeding [56], in line with its localization in the hypothalamus (Fig. 7 A-D; in [3])."[3]

1.6. Discussion

1.6.1. Characterization of the mouse habenula

Klüver–Barrera stain allows simultaneous visualization of neuronal cell bodies and myelinated fibers. Thus it was possible to identify known habenular subnuclei from the rat also in mice by characteristic topographic localization, as well as neuronal density and morphology (see [1], Fig. 5). "Unfortunately, the distributions of several cytochemical markers like the GABA-B receptor were found to be less reliable indicators of subnuclear areas."[1]

1.6.2. Methodical limitations of the Allen Brain Atlas and microarray analysis

"To analyse regionally enriched transcripts, three seed voxels (SVs) in the Allen Brain Atlas were selected within the borders of the LHb. In contrast, the retrieved transcripts were not restricted to the SV-areas, sometimes distributed all over the brain. This may be due to the fact that the system does not allow to define SVs within the borders of LHb subnuclei. Furthermore, the "Correlation-tool" in the Allen Brain database favors densely packed, highly expressed transcripts taking to little account of isolated and less prominent cells. Subsequently it was necessary to use neuroanatomical manual analysis to detect transcripts with interesting distribution patterns in the LHb or the MHb."[2] "For microarray analysis the impact of data also largely depends on the structural precision of sample aquisition. However, focusing on selected transcripts with known and characteristic expressions in MHb, LHb, or the thalamus (Table 2; in [3]), indicates an accurate microdissection procedure."[3]

1.6.3. Potential roles of habenular subnuclei in diverse biological functions

Microarray data indicate the presence of nicotinergic and muscarinergic AChRs in the habenula. Indeed, mRNA coding for the inhibitory mAChR2 (Table 5b; in [3]) is obvious in the LHb (Fig. 2 E; in [3]), especially in the area of the parvocellular subnucleus of the LHbM (Fig. 2 G; in [3]). As mAChR2 is functionally known as presynaptic auto- and heteroreceptor the immunoreactive area may represent incoming axon terminals. [3].

"Glutamatergic transmission is commonly thought to be excitatory. Excitatory metabotropic glutamate receptors like mGluR1 and mGluR5 are higher in the LHb as compared to the MHb (Table 5; in [3]). This is especially interesting, as these two receptors are known, to be part of the machinery of pain [57]. However, cells in the habenular area also contain inhibitory, metabotropic glutamate receptors like mGluR3 [58]. The mGlu3 receptor is known in presynaptic but also in somatodendritic localization [59] opening the possibility that glutamate release in the MHb and LHb also may result in local inhibition (Fig. 3; in [3])."[3] As recently found, also serotinergic input from raphe nuclei modulates glutamatergic transmission in lateral habenula [60], contributing a potential role of homestatic balance of serotinergic system and negative-reward processing.

"GABAergic transmission is commonly thought to be inhibitory. GABA-B activation suppresses LHb neuronal firing, indicating the habenular neuronal excitability partly under GABAb-receptor-mediated control [61]. But to increase the complexity, the activation of GABA-A receptors may result either in inhibition or in activation of the target neuron, depending on the intracellular chloride concentration (Cl_i) of the postsynaptic cell. A Cl_i of around 8 mM, which is common for many neurons, requires the pumping activity of the KCC2 potassium/chloride cotransporter [62]. In neurons without the KCC2 transporter, GABAergic transmission will be excitatory. Our data here indicate that the MHb largely is devoid of the KCC2 transporter (Fig. 4 G, Table 5 b; in [3]). These results may explain, why no inhibitory currents could be evoked by GABA administration and suggest that the fast GABAergic transmission in the MHb is largely excitatory. This does not mean that GABA acts exclusively excitatory, as the MHb also displays a high density of GABA-B receptors."[3]

"Very interesting is the localization of delta subunit expressing neurons in the LHb (Fig. 4 F; in [3]). This relies on the fact that "slow" GABAergic transmission is mediated by those GABA-A receptors containing the delta subunit. Current evidence suggests that a certain type of interneuron, the neurogliaform cell, mediate this type of inhibition [63]."[3]

1.6.4. Interpretation of potential markers for habenular subareas

„The present work provides data suggesting that the LHb neurons are composed of many distinct cell types with an unanticipated molecular heterogeneity. As expected, Gpr151 was among the retrieved transcripts. However, in contrast to previous reports [47, 48], we disclosed that this gene was not exclusively expressed in the MHb, but also in the LHb and that Gpr151 mRNA is not restricted to the habenular area. Consequently, behavioral investigations of transgenic animals, in which Gpr151-expressing neurons are selectively ablated by molecular biological techniques [34], must not be interpreted as animals with a selectively destroyed MHb. This also holds true for other transcripts, which in the literature have been considered as selective markers the habenula (see [2]). In contrast to our expectations, we did not find any gene [2, 3], which may be used as a novel marker to identify one of the known subnuclei in the LHb.

Neuronal cell types usually are classified according to their morphology, to their major transmitter, to their effect on target neurons, to their electrophysiology, to intracellular calcium-binding proteins, or to characteristic neuropeptides. Conceding that often there are fixed combinations of criteria, estimations result in potentially hundreds of distinct neuronal cell types [64]. Grouping about 140 habenula-enriched transcripts according to their distributions resulted in 9 separate patterns (Table 1 supplement; in [2]) and an additional group of transcripts, with individually distinct distributions (pseudopattern 10). Comparing the distributions of individual transcripts in detail, in some cases (see pattern 6) localizations overlap within the pattern groups. These or some of these transcripts might indeed characterize specific neuronal cell types. Such conclusions, however, have to await colocalizations on the mRNA and protein level.

In contrast, in the MHb many genes tended to respect subnuclear boundaries (Table 2; in [2]). Others exhibit conspicuous characteristic distributions, leading to the identification a novel subfield adjacent to the region of the MHbVm, which now is termed intermediate field of the ventral MHb. In addition, we identified a lateral extension of the MHb (HbX area), which is composed of intermingled MHb and LHb neurons and may allow functional interaction between the both habenular complexes. In several cases transcripts were restricted to individual subnuclear areas alone or in combinations (see Fig. 6; in [2]). This might may offer the possibility to create transgenic animals, in which these subnuclei are deleted as had been attempted earlier for the complete habenular area."[2]

1.6.5. Involvement of the habenula in homeostatic behaviors

"Quite surprisingly, neuronatin, the neuropeptide product of the Nnat gene, was among the top 100 highest transcripts in the habenula (supplemental Table 2; in [3]). It is found in feeding-

regulating hypothalamic nuclei, in pancreas, and in adipose tissue [56]. The high expression of the prokineticin 2 receptor is in line with a suspected role of the LHb during the circadian rhythm. Furthermore, several G-protein related receptors (Grp83, Grp139, Grp149, Grp151, Grp158) and many neuropeptides related to feeding are differentially expressed in the habenular region, indicating that the involvement of MHb and LHb in the regulation of food consumption and energy expenditure may have been underestimated so far."[3]

1.6.6. Potential involvement of the habenula in autism

"Habenular dysfunction often has been discussed in relation to severe neuropsychiatric disorders like schizophrenia or major depression [7, 65]. Both diseases are based on a polygenic background. In addition, the collection of genes investigated here contains a considerable number, which may be involved in autism spectrum disorders (ASD). Using hypergeometric tester (http://spark.rstudio.com/leonfrench/ hyper/) these known ASD-related transcripts are significantly (p = 0.002) enriched among habenular genes, indicating a potential involvement of the MHb and/or LHb complexes in ASD."[2]

1.6.6. Concluding remarks

The main objectives of an intense transcriptomic and morphological analysis were (I) to find additional marker of habenular subnuclei and (II) to find novel marker genes for neuronal subtypes to understand the biological function of the habenula in rat and mouse in more detail.
Thus borders of mouse habenular subnuclei had to be defined morphologically (see [1]). Fortunately the habenula in both species, rat and mice, basically look similar, so our work was to find out if they were homologous (Fig. 1 A-D, [1]). Using transcript-expression studies (see [3]), in-situ-hybridization (see [2, 3]) and histochemical analysis now in both, rat and mice resulted in

(1) detailed morphological characterization of habenula subnuclei in the mouse[1, 2],

(2) an unanticipated molecular heterogeneity of cell types in the habenula [2, 3],

(3) an increased awareness of the genetic and functional diversities of MHb and LHb (e.g. transcriptomic profile; differential expression of KCC2-transporter and thus potential different GABAergic signal transmission, or potentially inhibitory effects of acetylcholine and glutamate in the habenula [3]) and

(4) a deeper understanding of the habenular involvement in homeostatic regulation [3]including the possible involvement in autistic behavior [2].

All original datasets of this microarray study are given as open source. Further experiments will be needed to especially confirm the biological impact of described selective expressed proteins.

1.7. References

1 Wagner F, Stroh T, Veh RW. Correlating habenular subnuclei in rat and mouse by using topographic, morphological and cythochemical criteria. J Comp Neurol 2014;522:2650-62.

2 Wagner F, French L, Veh RW. Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries. Brain Struct Funct 2016; 221:39-58.

3 Wagner F, Bernard R, Derst C, French L, Veh RW. Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance. Brain Struct Funct 2016;[Epub ahead of print].

4 Schultz W. Predictive reward signal of dopamine neurons. J. of Neurophys 1998;80:1-27.

5 Olds J and Milner P. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. J Comp Physiol Psychol. 1954;47:419–27.

6 Shepard PD, Holcomb HH and Gold JM. Schizophrenia in translation: the presence of absence: habenular regulation of dopamine neurons and the encoding of negative outcomes. Schizophr Bull. 2006;32:417-21.

7 Li B, Piriz J, Mirrione M, Chung C-H, Proulx CD, Schulz D, Henn F and Malinow R. Synaptic potentiation onto habenula neurons in the learned helplessness model of depression. Nature. 2011;470:535-39.

8 Krach S, Paulus FM, Bodden M, Kircher T. The Rewarding Nature of Social Interactions. Front Behav Neurosci. 2010;4:22.

9 Berridge KC, Robinson TE, Aldridge JW. Dissecting components of reward: 'liking', 'wanting', and learning. Curr Opin Pharmacol. 2009;9:65-73.

10 Berridge KC. The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacol. 2007;191:391-431.

11 Meye FJ, Adan RAH. Feelings about food: the ventral tegmental area in food reward and emotional eating. Trends Pharmacol Sci 2014;35:31-40.

12 Schultz W. Dopamine neurons and their role in reward mechanisms. Curr Opin Neurobiol. 1997;2:191-7.

13 Christoph GR, Leonzio RJ, Wilcox KS. Stimulation of the lateral habenula inhibits dopamine-containing neurons in the substantia nigra and ventral tegmentum area of the rat. J Neurosci 1986;6:613-619.

14 Ji H, Shepard PD. Lateral Habenula Stimulation Inhibits Rat Midbrain Dopamine Neurons through a GABA-A Receptor-Mediated Mechanism. J Neurosci 2007;27:6923-30.

15 Lisoprawski A, Herve D, Blanc G, Glowinski J, Tassin JP. Selective activation of the mesocortico-frontal dopaminergic neurones induced by lesions oft he habenula in the rat. Brain Res. 1980;183:229-34.

16 Matsumoto M, Hikosaka O. Lateral habenula as a source of negative reward signals in dopamine neurons. Nature 2007;447:1111-15.

17 Craige E. An introduction to the finer anatomy of the central nervous system based upon that of the albino rat. Univ. Toronto. Press. 1925.

18 Marburg O, The structure and fibre connections of the human habenula. J. Comp. Neur. 1944;80:211-34.

19 Herkenham M, Nauta WJH.Afferent connections of the habenular nuclei in the rat. A horseradish peroxidase study, with a note on the fiber-of-passage problem. J Comp Neurol. 1977;173:123-46.

20 Shabel SJ, Proulx CD, Trias A, Murphy RT, Malinow R. Input to the Lateral Habenula from the Basal Ganglia Is Excitatory, Aversive, and Suppressed by Serotonin. Neuron. 2012;74:475-81.

21 Contestabile A, Fonnum F. Cholinergic and GABAergic forebrain projections to the habenula and nucleus interpeduncularis: surgical and kainic acid lesions. Brain Res. 1983;275:287–97.

22 Gruber C, Kahl A, Lebenheim L, Kowski A, Dittgen A, Veh RW. Dopaminergic projections from the VTA substantially contribute to the mesohabenular pathway in the rat. Neurosci Lett. 2007;427:165-70.

23 Qin C, Luo M. Neurochemical phenotypes of the afferent and efferent projections of the mouse medial habenula. Neuroscience. 2009;161:827-37.

24 Omelchenko N, Bell R, Sesack SR; Lateral habenula projections to dopamine and GABA neurons in the rat ventral tegmental area. Eur J Neurosci. 2009;30:1239-50.

25 Lammel S, Ion DI, Roeper J, Malenka RC. Projection-Specific Modulation of Dopamine Neuron Synapses by Aversive and Rewarding Stimuli. Neuron 2011;70:855-62.

26 Bernard R, Veh RW. Individual Neurons in the Rat Lateral Habenular Complex Project Mostly to the Dopaminergic Ventral Tegmental Area or to the Serotonergic Raphe Nuclei. J Comp Neurol. 2012;520:2545-58.

27 Goncalves L, Sego C, Metzger M. Differential Projections From the Lateral Habenula to the Rostromedial Tegmental Nucleus and Ventral Tegmental Area in the Rat. J Comp Neurol 2012; 520:1278-1300.

28 Semba K, and Fibiger HC. Time of origin of cholinergic neurons in the rat basal forebrain. J. Comn. Neurol. 1988;269:87-95.

29 Nair-Roberts RG, Chatelain-Badie SD, Benson E, White-Cooper H, Bolam JP, Ungless MA. Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. Neuroscience. 2008;152:1024–1031.

30 Brinschwitz K, Dittgen A, Madai VI, Lommel R, Geisler S, Veh RW. Glutamatergic axons from the lateral habenula mainly terminate on GABAergic neurons of the ventral midbrain. Neurosci. 2010;168:463-76.

31 Stamatakis AM, Jennings JH, Ung RL, Weinberg RJ, Neve RL, Boyce F, Mattis J, Ramakrishnan C, Deisseroth K and Stuber GD. A unique population of ventral tegmental area neurons inhibits the lateral habenula to promote reward. Neuron. 2013;80:1039-53.

32 Herkenham M, Nauta WJH. Efferent Connections of the Habenular Nuclei in the Rat. J Comp Neurol. 1979;18:19-48.

33 Salas R, Sturm R, Boulter J, De Biasi M. Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. J. Neurosci. 2009;29:3014–18.

34 Kobayashi Y, Sano Y, Vannoni E, Goto H, Suzuki H, Oba A, Kawasaki H, Kanba S, Lipp H-P, Murphy NP, Wolfer DP, Itohara S. Genetic dissection of medial habenula -interpeduncular nucleus pathway function in mice. Front Behav Neursci 2013;7:1-20.

35 Corodimas KP, Rosenblatt JS, Canfield ME, Morrell JI. Neurons in the lateral subdivision of the habenular complex mediate the normal onset of maternal behavior in rats. Behav Neurosci. 1993;5: 827-43.

36 Shumake J, Edwards E, Gonzalez-Lima F. Opposite metabolic changes in the habenula and ventral tegmental area of a genetic model of helpless behavior. Brain Res. 2003;963:274-81.

37 Cohen SR, Melzack R. Habenular stimulation produces analgesia in the formalin test. Neurosci Lett. 1986; 25;70:165-69.

38 Hikosaka O. The habenula: from stress evasion to value-based decision-making. Nat Rev Neurosci. 2010;11:503-13.

39 Görlich A, Antolin-Fontes B, Ables JL, Frahm S, Slimak MA, Dougherty JD, Ibañez-Tallon I. Reexposure to nicotine during withdrawal increases the pacemaking activity of cholinergic habenular neurons. Proc Nat Acad Sci USA 2013;110:17077-82.

40 Yamaguchi T, Danjo T, Pastan I, Hikida T, Nakanishi S. Distinct roles of segregated transmission of the septo-habenular pathway in anxiety and fear. Neuron. 2013;78:537-44.

41 Hsu YW, Wang SD, Wang S, Morton G, Zariwala H, de la Iglesia HO, Turner EE. Role of the dorsal medial habenula in the regulation of voluntary activity, motor function, hedonic state, and primary reinforcement. J Neurosci. 2014;34:11366-84.

42 Geisler S, Andres KH, Veh RW. Morphologic and cytochemical criteria for the identification and delineation of individual subnuclei within the lateral habenular complex of the rat. J Comp Neurol. 2003;458:78-97.

43 Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNAsequencing and microarray studies. Nucleic Acids Res. 2015;43:1-13.

44 Speel EJ and Komminoth P. CARD In Situ Hybridization: Sights and Signals. Endocr Pathol. 1999;10:193-98.

45 Madai V. Virtual pre-embedding labeling, a method for correlative fluorescence and electron microscopic imaging and double-labeling in the central nervous system of the rat. Diss. 2012; http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000037352

46 Ng L, Pathak S, Kuan L, Lau C, Dong HW et al. Neuroinformatics for 3-D Gene Expression Mapping in the Mouse Brain. Trans. Comput. Biol. Bioinf. 2007;4:382-93.

47 Berthold M, Collin M, Sejlitz T, Meister B, Lind P. Cloning of a novel orphan G protein-coupled receptor (GPCR-2037): in situ hybridization reveals high mRNA expression in rat brain restricted to neurons of the habenular complex. Mol. Brain Res. 2003;120:22-29.

48 Aizawa H, Kobayashi M, Tanaka SC, Fukai T, Okamoto H. Molecular Characterization of the Subnuclei in Rat Habenula. J Comp Neurol. 2012;520:4051-66.

49 Gras C, Herzog E, Bellenchi GC, Bernard B, Ravassard P, Pohl M et al. A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J Neurosci 2002; 22:5442-51.

50 Herzog E, Gilchrist J, Gras C, Muzerelle A, Ravassard P, Giros B et al. Localization of vglut3, the vesicular glutamate transporter type 3, in the rat brain. Neuroscience. 2004;123:983-1002.

51 Gruber C, Kahl A, Lebenheim L, Kowski A, Dittgen A, Veh RW. Dopaminergic projections from the VTA substantially contribute to the mesohabenular pathway in the rat. Neurosci Lett. 2007;427:165-170.

52 Good CH, Wang H, Chen YH, Mejias-Aponte CA, Hoffman AF, Lupica CR. Dopamine D4 receptor excitation of lateral habenula neurons via multiple cellular mechanisms. J. Neurosci 2013; 43:16853-64.

53 Han LN, Zhang L, Li LB, Sun YN, Wang Y, Wang Y et al. Activation of serotonin receptors in the lateral habenular nucleus increases the expression of depression-related behaviors in the hemiparkinsonian rat. Neuropharm. 2015;93:68-79.

54 Boschert U, Amara DA, Segu L, Hen R. The Mouse 5-Hydroxytryptamine(1B) Receptor Is Localized Predominantly on Axon Terminals. Neurosci. 1994;58:167-82.

55 Hwang EK, Chung JM. 5HT(1B) receptor-mediated pre-synaptic depression of excitatory inputs to the rat lateral habenula. Neuropharmacology. 2014;81:153-65.

56 Vrang N, Meyre D, Froguel P, Jelsing J, Tang-Christensen M, Vatin V, et al. The Imprinted Gene Neuronatin Is Regulated by Metabolic Status and Associated With Obesity. Obesity 2010;18:1289-96.

57 Radulovic J, Tronson NC. Preso1, mGluR5 and the machinery of pain. Nat Neurosci. 2012;15:805-7.

58 Petralia RS, Wang YX, Niedzielski AS, Wenthold RJ. The metabotropic glutamate receptors, mGluR2 and mGluR3 show unique postsynaptic, presynaptic, and glial localizations. Neuroscience 1996;71:949-976.

59 Tamaru Y, Nomura S, Mizuno N, Shigemoto R. Distribution of metabotropic glutamate receptor mGluR3 in the mouse CNS: Differential location relative to pre- and postsynaptic sites. Neuroscience. 2001;106:481-503.

60 Xie G, Zuo W, Wu L, Li W, Wu W, Bekker A, Ye H. Serotonin modulates glutamatergic transmission to neuron in the lateral habenula. Scientific reports. 2016;6:23798.

61 Lecca S, Pelosi A, Technio A, Moutkine I, Lujan R, Hervé D, Mameli M. Rescue of GABAB and GIRK function in the lateral habenula by protein phosphatase 2A inhibition ameliorates depression-like phenotypes in mice. Nature medicine 2016;22:254-61.

62 Chamma I, Chevy Q, Poncer JC, Levi S. Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission. Front Cell Neurosci.20126:1-15.

63 Tamas G, Lorincz A, Simon A, Szabadics J. Identified sources and targets of slow inhibition in the neocortex. Science. 2003;299:1902-5.

64 Sugino K, Hempel CM, Miller MN, et al. Molecular taxonomy of major neuronal classes in the adult mouse forebrain. Nature Neuroscience. 2006;9:99-107.

65 Sartorius A, Kiening KL, Kirsch P, Gall CC, Haberkorn U, Unterberg AW et al. Remission of major depression under deep brain stimulation of the lateral habenula in a therapy-refractory patient. Biol Psychiatry. 2010;67:e9-e11.

3. Anteilserklärung / eidesstattliche Erklärung

„Ich, Franziska Wagner, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation: "Genomic-morphological analysis of the rat and mouse habenulae uncovers a high molecular heterogeneity and indicates involvement in the regulation of feeding and energy balance" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)" des ICMJE -*www.icmje.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

____

Franziska Wagner　　　　　　　　　　　　　　　　　　　　Berlin, den 21.06.2016

Anteilserklärung an den erfolgten Publikationen

Franziska Wagner hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: [Wagner F, Stroh T, Veh RW], [Correlating habenular subnuclei in rat and mouse by using topographic, morphological, and cytochemical criteria], [J. Comp Neurol], [2014]

Beitrag im Einzelnen: F.W. leistete einen wesentlichen Beitrag bei der Zusammenstellung der Versuche, morphologischen Analyse der Unterkerne, bei Erstellung des Manuskripts und der Grafiken.

Publikation 2: [Wagner F, French L, Veh RW], [Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries], [Brain Struct Funct], [2016]
Beitrag im Einzelnen: F.W. leistete einen wesentlichen Anteil in der Konzeption der Versuche synthetisierte die Riboproben, Durchführung der immunhistochemischen und In-Situ-Hybridisierungsversuche, Auswertung der Allen Brain Atlas Daten und Erstellung des Manuskripts und der Grafiken.

Publikation 3: [Wagner F, Bernard R, Derst C, French L, Veh RW], [Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance], [Brain Struct Funct], [2016]
Beitrag im Einzelnen: F.W. leistete einen wesentlichen Beitrag bei der Konzeption und Auswertung der Microarray-Versuche, der Literaturrecherche zur Auswertung der Datensätze, Durchführung der immunhistochemischen und In-Situ-Hybridisierungsversuche, Erstellung des Manuskripts und der Grafiken.

Prof. em. Rüdiger W. Veh Berlin, den 21.06.2016

Franziska Wagner Berlin, den 21.06.2016

3. Published Manuscripts

3.1. Correlating habenular subnuclei in rat and mouse by using topographic, morphological, and cytochemical criteria

3.2. Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries

3.3. Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance

3.1. Correlating habenular subnuclei in rat and mouse by using topographic, morphological, and cytochemical criteria

Citation:

Wagner F, Stroh T, Veh RW. Correlating habenular subnuclei in rat and mouse by using topographic, morphological, and cythochemical criteria. J. Comp Neurol 2014;522:2650-62.

http://dx.doi.org/10.1002/cne.23554

3.2. Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries

Citation:

Wagner F, French L, Veh RW. Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries. Brain Struct Funct 2016;221:39-58.

http://dx.doi.org/10.1007/s00429-014-0891-9

3.3. Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance

Citation:

Wagner F, Bernard R, Derst C, French L, Veh RW. Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance. Brain Struct Funct 2016;221:4663-4689.

http://dx.doi.org/10.1007/s00429-016-1195-z

4. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

5. Bibliography

5.1. Publications

Wagner F, Stroh T, Veh RW. Correlating habenular subnuclei in rat and mouse by using topographic, morphological, and cythochemical criteria. J. Comp Neurol 2014;522:2650-62. Impact Factor 3.2

Wagner F, French L, Veh RW. Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries. Brain Struct Funct 2016;221:39-58. Impact Factor 5.6

Wagner F, Bernard R, Derst C, French L, Veh RW. Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance. Brain Struct Funct 2016;221:4663-4689. Impact Factor 5.6

5.2. Poster:

Wagner F, Bernard R, Derst C, French F, Veh RW, Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance. 2015. Annual Meeting of the Society for Neuroscience; Chicago

Wagner F and Veh RW, A genomic anatomic approach to identify novel markers for potentially distinct neuronal cell types within the lateral habenular complex of the adult mouse. 2013. Annual Meeting of the Society for Neuroscience; SanDiego

Wagner F, Derst C, Veh RW, Subnuclear distribution of neurons selectively expressing genes in the rat. 2011. Annual Meeting of the Society for Neuroscience; Washington, DC

Wagner F, Derst C, Veh RW. Microarray analysis of habenula: identification of cDNAs differentially expressed in the medial and lateral habenular complexes and in the thalamus of the rat. 2011. 9th Göttingen Meeting of the German Neuroscience Society

6. Acknowledgements

Working in the group of Rüdiger W. Veh gave me the opportunity to learn and to work independently. I would like to thank Rüdiger W Veh for teaching us to go right to the bottom of things and do not stop asking on common accepted paradigms. I am grateful for those of you who stood by to support me along the way, especially I want to thank Leon French, Christian Derst and René Bernard.

I would like to thank Ina Wolter, Heike Heilmann and Anne Löchner for excellent technical assistance. Especially I want to thank Annett Kaphahn for administrative help and constructive advices through the jungle. The financial support of the FAZIT foundation is gratefully acknowledged.

Finally I would like to thank my mother, who now is quite well informed about the habenula.