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Individual neurons in the rat lateral habenular complex mostly project to either the dopaminergic ventral tegmental area or to the serotonergic raphe nuclei

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Running head: LHb efferent projections to raphe nuclei and VTA

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Abstract

The lateral habenular complex (LHb) is a bilateral epithalamic brain structure involved in the modulation of ascending monoamine systems in response to afferents from limbic regions and basal ganglia. The LHb is implicated in various biological functions, such as reward, sleep-wake cycle, feeding, pain processing and memory formation. The modulatory role of the LHb is partly assumed by putative spontaneously active LHb neurons projecting to the dopaminergic ventral tegmental area (VTA), and to the serotonergic median (MnR) and dorsal raphe nuclei (DR). All four nuclei form a complex and coordinated network to evoke appropriate responses to reward-related stimuli.

At present it is not known whether individual LHb neurons project to only one or to more than one monoaminergic nucleus. To answer this question we made dual injections of two different retrograde tracers into the rat VTA and either DR or MnR. Tracers were visualized by immunocytochemistry. In coronal sections the different retrogradly habenular labeled neurons were quantified and assigned to the corresponding habenular subnuclei.

Our results show that (1) the distribution of neurons in the LHb projecting to the three monoamine nuclei is similar and exhibits a great overlap, (2) the vast majority of LHb projection neurons target one monoaminergic nucleus only, and (3) very few, heterogeneously distributed LHb neurons project to both, dopaminergic and serotonergic nuclei.

These results imply that the LHb forms both, separate and interconnected circuits with each monoaminergic nucleus, permitting the LHb to modulate its output to different monoamine systems either independently or conjointly.

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Introduction

The bilateral habenular nucleus of the epithalamus consists of a medial (MHb) and a lateral (LHb) complex. Both parts receive input from structures such as lateral hypothalamus, lateral preoptic area, entopeduncular nucleus and basal ganglia via the stria medularis and the medial forebrain bundle (Herkenham and Nauta, 1977; Kowski et al., 2008). The fasciculus retroflexus serves as the main output of the habenula, with MHb efferents projecting to the interpeduncular nucleus (IPN) while LHb neurons, among others, target almost every major monoaminergic cell group of the brainstem (Herkenham and Nauta, 1979) to modulate the release of different monoamine and non-monamine neurotransmitters.

The biological importance of the LHb has been understood only recently, when its major role as the center of an "anti-reward system" was recognized (Ullsperger and von Cramon, 2003; Shepard et al., 2006; Matsumoto and Hikosaka, 2008). Unsuccessful and dissatisfying behavior causes increased LHb activity (Matsumoto and Hikosaka, 2007). Electrical stimulation of the LHb suppresses the activity of dopaminergic neurons in the VTA (Christoph et al., 1986; Ji and Shepard, 2007) and serotonergic cell groups in DR and MnR (Wang and Aghajanian, 1977; Stern et al., 1979). Such inhibition is achieved through glutamatergic efferents from the LHb that target GABAergic neurons within the monoaminergic nuclei (Brinschwitz et al., 2010; Omelchenko et al., 2009). Together with its efferent targets VTA, DR, and MnR, the LHb acts as a central relay nucleus to form a complex and coordinated network for processing of emotion- and motivation-encoded information (Hikosaka, 2010). In addition, the LHb is known to regulate complex behaviors such as cognition, attention, pain processing, sleep-wake rhythm, and stress response (Lecourtier and Kelly, 2007; Klemm 2004; Sutherland 1982), all of which involve a large degree of monoaminergic regulation.

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Consistently, the LHb presents a complex morphological organization into ten individual subnuclei, which might represent different functional entities (Andres et al., 1999; Geisler et al, 2003; Kowski et al., 2009; Li et al., 2011). Due to its central role in monoamine regulation evidence is mounting that the habenula is implicated in the etiology of different psychiatric disorders, including major depression (Sartorius et al., 2010; Li et al., 2011; Winter et al., 2011) and schizophrenia (Sandyk, 1992; Ellison, 1994; Lecourtier et al., 2004). Recent data from our group (Poller et al., 2011) and others (Lecourtier et al, 2008) suggests that the modulatory role of the LHb is partly assumed by putative spontaneously active neurons that exert a tonic inhibition of monoaminergic activity in the dopaminergic ventral tegmental area (VTA), and the serotonergic median (MnR) and dorsal raphe (DR) nuclei.

VTA, DR and MnR receive habenular input by neurons located in the LHb (Herkenham and Nauta, 1979). Thus, the LHb is in a position to control the output of different monoaminergic groups that provide dopamine and serotonin to the forebrain. The construct of parallel, functionally similar LHb efferents to monaminergic nuclei gives rise to possibility that one single LHb neuron might project to cells in more than one monoamine nucleus (Figure 1B). Alternatively, individual neurons or neuronal groups in the LHb may selectively project to only one of the monoaminergic nuclei (Figure 1A).

In the present investigation two different retrograde tracers sequentially were microinjected into the VTA and DR or MnR of individual rats to differentiate between these two hypotheses. Series of coronal sections were used for quantification of double labeled neurons within the boundaries of the LHb.

Material and Methods

Chemicals were obtained from Sigma-Aldrich, Taufkirchen, Germany, if not indicated otherwise. All animal experiments were approved by the Regional Berlin Animals Ethics Committee and conducted in strict accordance with the European Communities Council

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directive regarding care and use of animals for experimental procedures. Adult, male Wistar rats weighting 250–360 g were obtained from an institutional breeder (Forschungs-einrichtungen für Experimentelle Medizin, Charité-Universitätsmedizin Berlin, Krahmerstr. 6–10, D-12207 Berlin, Germany). Animals were housed in group-cages under controlled temperature (22 °C) and illumination (12 h light/dark cycle) conditions with food and water *ad libitum*.

Tracer injections

Animals were placed in a plexiglass chamber in which anesthesia was induced with 5% isoflurane (in 100% O_2). Anesthetized rats then were placed in a ~~stereotaxic~~ stereotactic frame (David Kopf, Tujunga, CA), and isoflurane was delivered through a rat anesthesia mask (David Kopf) using a flow rate of 0.6 liters of O_2/min. Core body temperature was monitored and maintained using a feedback-controlled heating pad set at 37°C. Using a small drill (Proxxon, Niersbach, Germany), one hole was made in the skull to permit unilateral access to the anterior brainstem according to a rat brain atlas (Paxinos and Watson, 1998). For tracer injection, the following ~~stereotaxic~~ stereotactic coordinates were used, for the VTA: 6.0 mm posterior to bregma, 0.5 mm from the midline, and 7.8 mm ventral to the dura; for the DR: 7.8 mm posterior to bregma, 0.9 from the midline, and 5.7 mm ventral to the dura (-8°); for the MnR: 7.8 mm posterior to bregma, 0.8 from the midline, and 7.8 mm ventral to the dura (-6°). Hemisphere for injections into VTA was randomized prior to each experiment. The retrograde tracer Fluorogold (FG; 2-hydroxy-4,4-diamino-stilbene, 1% in 0.1 M cacodylate buffer, pH 7.4; Fluorochrome, Denver, CO, USA) was either injected into the MnR or the DR of the rat. In addition, all rats received an unilateral injection of the retrograde tracer gold-coupled wheatgerm agglutinin (WGA-apoHRP-gold, 15 nm particle size; 10–20 µg/ml in distilled H_2O, pH 7.0–7.5, E-Y Laboratories, San Mateo, CA, USA) into the VTA.

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FG was iontophoretically injected through 1.0 mm glass micropipettes pulled to tip diameters of 20 µm over 10-15 minutes at an amperage of 1 µA in 7 second intervals, whereas 200-300 nl of WGA-apoHRP-gold was pressure-injected using 1.0-mm glass pipettes pulled to outer tip diameters of 30–40 µm. After the injection was completed, micropipettes were left in situ for another 5 minutes to minimize spread of the tracer. Next, pipettes were removed from the brain and the scalp was closed with surgical sutures. Anesthesia was discontinued, and after recovery rats were transfered to the their home cages.

Perfusion fixation

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After 4 days of survival rats were deeply anaesthetized by intraperitoneal injection of a mixture of 45 volume fractions ketamine hydrocholoride (100 mg/ml; DeltaSelect, Germany), 35 volume fractions xylazine hydrochloride (20 mg/ml, BayerVital, Germany), and 20 volume saline. This anesthesia mixture was dispensed at a dose of 0.16 ml/100 g body weight. In addition, 200 IU heparin sodium (Heparin-Rotexmedica; Trittau, Germany) were administered intraperioneally to prevent blood clotting during initial surgery and perfusion. Anesthesized rats were transaortically flushed for 10 seconds with 38°C warm plasma substitute (Deltadex 60; DeltaSelect, Germany), followed by 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid for 20 minutes. After a final 5 minute flush of the vasculature with 0.15 M sucrose in 0.1 M phosphate buffer (pH 7.4), brains were removed, immersed in 0.8 M sucrose overnight for cryoprotection, shock frozen in hexane at -70°C, and subsequently stored at -80°C until use. Brain blocks containing VTA, DR, MnR, and LHb were cut on a cryostat at -20°C into 10 series of 25 µm coronal sections.

Silver Enhancement

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Coronal 25 µm thick sections were incubated in 10% sodium thioglycolate in 0.1 M phosphate buffer, pH 7.4 (PB) for 30 min and rinsed in 0.15 M sodium nitrate. Sections were then immersed for 2 times 20 min in a mixture of solutions A and B of the IntenSE M Silver Enhancement Kit (GE Healthcare, München, Germany). Between incubations sections were rinsed for 5 min in 0.15 M sodium nitrate. After the second 20 min immersion, residual silver ions were removed by fixation for 10 min in 5% sodium thiosulfate in PB.

Immunohistochemistry

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Free floating brain sections that underwent silver enhancement were rinsed in phosphate buffered saline (PBS; 0.15 M sodium chloride in 0.01 M phosphate buffer, pH 7.4). After 15 min incubation with 1% sodium borohydride in PBS, and thoroughly washing in PBS, sections were pretreated for 30 minutes in a solution containing 10% normal goat serum (NGS; Interchem, Germany), 0.3% Triton X-100 (Serva, Germany) and 0.05% phenylhydrazine (Merck, Germany), before being transferred to the primary antibody solution containing rabbit anti-FG antibodies at 1:10,000 dilution (Millipore, Billerica, MA, USA), in 10% NGS, 0.3% Triton, 0.1% sodium azide, and 0.1% thimerosal for 48 hours at 4°C. After washing twice in PBS and 60 minutes of preincubation in PBS-A (2 mg/ml bovine serum albumin in PBS), sections were exposed for 24 hours at 4° C to the secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), dilution 1:2,000 in PBS-A with 0.3% Triton and 0.1% sodium azide. Subsequently, sections were repeatedly rinsed in PBS, preincubated in PBS-A, and treated for 12 hours with a peroxidase-streptavidin solution (1:10,000 in PBS; Perkin Elmer, Rodgau-Jügesheim, Germany). Sections were washed in PBS, preincubated for 15 min in a 1.4 mM 3,3-diaminobenzidine (DAB, 0.5 mg/ml) solution containing 10 mM imidazol in 0.05 M Tris buffer, pH 7.6. The color reaction was developed by adding hydrogen peroxide to the DAB solution to a final concentration of 0.0015% and was stopped after 15 minutes by repeated washes with PBS. Sections were

mounted onto gelatin-coated glas slides, air dried, dehydrated through a graded series of ethanol, transferred into xylene (J.T. Baker, Holland), and coverslipped with Entellan (Merck, Germany).

Antibody specificity

The primary antibody used in this study came from an antiserum against the retrograde tracer FG (Chemicon, Temecula, CA; catalog No. AB153, lot 21081641) and was originally raised in rabbit against hydroxystilbamidine, linked to bovine serum albumin and keyhole limpet hemocyanin via a mixed aldehyde fixative (Chang et al., 1990). The specific of this antibody was confirmed from reactions with brain sections obtained from animals that had not received any tracer injection. These sections were without labeling.

Klüver-Barrera stain

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Free-floating 25 µm sections were rinsed in PBS, followed by extraction of lipids in 70% ethanol for 12 hours as previously described (Geisler et al., 2002). Myelinated fibers were stained overnight at 56°C in a 0.1% Luxol fast blue solution in 96% ethanol and 0.05% acetic acid. Thereafter, sections were washed in distilled water and differentiated for 3 minutes in 0.01% lithium carbonate. Subsequently, sections were transferred to 70% ethanol for 3 minutes and washed again. For subsequent cresyl violet stain, slides were washed for 2 min in distilled water and transferred for 30 min into the cresyl violet acetate solution containing 0.2% cresyl violet in 20 mM acetate buffer at the appropriate pH value (Geisler et al., 2002). After rinsing in distilled water, sections were dehydrated fast and coverslipped.

Digitization and data analysis

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Twelve animals were rejected for further study because tracer spread beyond the boundaries in one of the two target sites. Only animals (n=6) with satisfactory dual tracer injection within the borders of VTA and MnR or DR were used for further analysis and quantification.

All light microscopic images were obtained with upright Leica DMRB light microscope connected to a high-resolution digital camera (MBF-CX9000, MBF Bioscience). Primary photographic data were adjusted for brightness, color, and contrast in Adobe Photoshop CS3 and arranged using Adobe Illustrator CS3.

Somata of retrogradely labeled habenular neurons were counted in sections regularly spaced at 250 µm along the anterior-posterior extent of the habenula. To assess overcounting error we employed the Abercrombie correction method (Abercrombie, 1946; Kowski et al., 2008). The resulting Abercrombie correction factor was 0.467 for 25 µm sections which indictates an overcount of 117%. Sections containing the LHb were identified and visualized with Neurolucida 8.0 software (MBF Bioscience, Williston, VT) through an upright Leica DMRB light microscope equipped with a high-resolution digital camera (MBF-CX9000, MBF Bioscience), a mechanical stage (Ludl, Thornwood, NY), and an x-y-z axis encoder connected to a PC running Windows XP Professional. Each habenula was outlined in tracing modus under 40x magnification. Neuronal somata that contained at least 3 distinctive tracer granules were counted and marked as “retrogradely labeled neuron”. Tagged maps of each habenula section were converted into TIFF Images using Neurolucida Explorer. Images of retrogradely labeled habenular neurons were overlaid in Adobe Photoshop CS3 with imaged from adjacent, Klüver Barrera stained sections, which aided the delineation of the subnuclear organization of the habenular complex. Another series was processed sections for Kir3.2 potassium channel immunocytochemistry (Geisler et al., 2003), which provided an improved localization of the LHbLO subnucleus. These procedures permitted the assignment of traced habenular neurons to their corresponding subnuclei.

Results

Microinjections of fluorogold (FG) into the DR result in medium to strong labeling in the habenula with the majority (>95%) of FG-containing neurons in the LHb (Fig. 2). Injections were placed along the dorsoventral extent of DR without spread into the neighboring ventrolateral periaqueductal gray (Fig. 3). Most intensive labeling is found toward the posterior end of the habenula and fewer traced neurons are located in the anterior part (Fig. 2A'-F'). Subnuclear analysis reveals that FG-labeled neurons stemming from DR microinjection are unevenly distributed across the LHb. The majority of labeling (68.5%) is almost equally distributed among three subnuclei (Fig. 4A): the parvocellular subnucleus of the medial division of the LHb (LHbMPc), the central subnucleus of the medial division of the LHb (LHbMC), and the magnocellular subnucleus of the lateral subdivision of the LHb (LHbLMc). Each of the remaining seven LHb subnuclei and the MHb present less than 7% of all retrogradely labeled DR projecting neurons.

Microinjections of FG into MnR produce wider distributed and more intensely stained habenular neurons than DR injections (Fig. 5A'-F'). Deposition of injected tracer was primarily concentrated in the MnR with minimal paramedian spread (Fig. 3). As for retrograde labeling from the DR the majority of neurons retrogradely marked by MnR injection are located in the posterior portion of the habenula (Fig. 5A'-F') and within the LHb. The majority of these neurons (58.4%) is localized to the same three subnuclei as in the case of the DR. However, LHb neurons retrogradely labeled from the MnR are unevenly distributed among the three subnuclei in contrast to those from the DR (Fig. 4B). Retrograde labeling is most prominent in the LHbLMc (30.9%), followed by LHbMC (16.6%), and LHbMPc (10.9%). Each of the remaining seven subnuclei present less than 10% of all MnR-based retrogradely labeled habenular neurons.

Retrograde tracer injections within the borders of the VTA result in medium to strong labeling of the LHb. VTA injections covered mainly the paranigral or parabrachial pigmented nuclei

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of the VTA almost throughout its entire rostrocaudal extent (Fig. 3). Contrary to labelings from raphe injections, the anterior-posterior distribution of retrogradely labeled neurons from VTA injections is relatively even (Fig. 4D). Consequently, retrogradely labeled LHb neurons now are distributed over four subnuclei: the anterior subnucleus of the medial subdivision of the LHb (LHbMA;16.1%), the LHbMC (14.8%), the LHbMPc (11.2%), and the LHbLMc (26.3%). Each of the other habenular subnuclei present less than 10% of VTA-based retrogradely labeled neurons (Fig. 4C).

In double retrograde tracing studies a population of habenular neurons was identified that contained retrograde tracers from both, the VTA and of one of the raphe nuclei (Table 1 and Fig. 6). However, only a minority of habenular neurons only presents both tracer substances: 1.3% of all labeled neurons from VTA and MnR tracer injections and 1.2% of all labeled neurons from VTA and DR tracer injections. Distribution of neurons within the habenula that contain both tracer substances follows the general pattern obtained from single injections into DR, MnR, or VTA.

To probe for the specificity of our retrograde labeling studies we performed control injections that targeted areas near the monoaminergic aim sites. An injection into periaqueductal gray, which is dorsolaterally situated from the DR aim site, did not produce retrograde labeling in the habenula (Fig. 7 A-B). Likewise, an injection dorsal from the VTA that was centered at the red nucleus resulted in little to no labeling in the habenula (Fig. 7 C-D). A microinjection lateral from the MnR, which included part of the paramedian raphe and the pontine reticular nucleus, oral part yielded a significant number of retrogradely labeled neurons. However, in opposite to MnR injections the vast majority of retrogradely labeled neurons were then located in the centro-lateral part of the LHb (Fig. 7 E-F), as previously described (Herkenham and Nauta, 1979; Kim, 2009). These control experiments demonstrate that retrograde tracer injections outside the three monoaminergic target areas either result in no habenular labeling or a completely different LHb labeling pattern and thus confirm the specificity of our results.

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Taken together we conclude that more than 98% LHb neurons that send descending projections to dopaminergic and serotonergic nuclei target only one nucleus, either the DR, the MnR, or the VTA. In addition, there is a small number of LHb neurons that provide axon terminals to more than one monoaminergic nucleus.

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Discussion

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The present study characterizes the efferent projections from the lateral habenular complex (LHb) to three major monoaminergic brainstem nuclei through dual retrograde tracer delivery. We quantified retrogradely traced neurons and assign their location to previously defined subnuclei of the LHb. Our analyses show that neurons projecting to MnR, DR, or VTA exhibit a very similar subnuclear distribution within the LHb. Retrogradely labeled neurons from the same three LHb subnuclei, the LHbLMc, the LHbMC, and the LHbMPc, account for more than half of all labeled neurons from microinjections into one monoamine target nucleus. Retrogradely labeled neurons from the raphe injections and VTA injections were differently distributed within the LHb, with respect to their rostro-caudal position. While LHb neurons projecting to DR and MnR are mainly located at the caudal end of the LHb, VTA projecting LHb neurons are rather evenly distributed from the rostral to the caudal portion of the LHb. We also identified a small population of LHb neurons that contained tracer granules from the VTA and one of the raphe microinjections. This suggests that few LHb neurons may be capable of simultaneously projecting to two different monoaminergic nuclei. The vast majority of LHb efferent neurons, however, forms independent connections with only one of the three investigated monoaminergic target nuclei.

Methodological considerations

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Retrograde tracers are aimed to be taken up by axons and then transported back to the neuronal somata, where tracer granules accumulate. Despite all the necessary care, two

circumstances cannot be excluded. First, spread of the tracer outside of the targeted nucleus and second, injections into fibers of passage. Therefore, we carefully evaluated each injection site and tracer spread using a rat brain atlas (Paxinos and Watson, 1998). Only experiments in which both tracer injections per rat were within the targeted nuclei were used for further analysis (Fig. 5). Injections can injure passing fibers resulting in false-positive labeling of certain neurons. Because fibers of all three nuclei join the fasciculus retroflexus, such a scenario cannot be excluded. We took several measures to reduce the likelihood for that to occur: First, we used glass capillaries with a small diameter and with a blunted tip. Second, we lowered the tracer-loaded tip into the brain with constant minimal speed in 50 µm increments. And thirdly, we injected only a small tracer amount, if possible by iontophoresis to reduce tissue damage and spread of tracer. In addition, all injection sites were evaluated for potential tissue damage.

In all tracer injection experiments we located retrogradely labeled neurons in the MHb which represented less than 10% of all labeled neurons. In general, the MHb is not regarded as a nucleus that projects to monoaminergic neurons. However, PHAL-tracer injection in the MHb shows moderate anterograde labeling in the MnR, the VTA, and near the lateral borders of the DR (Herkenham and Nauta, 1979). A retrograde tracing study confirmed that the MHb sends some projections to the MnR (Marcinkiewicz et al., 1989) whereas retrograde tracer injection into DR did not produce MHb labeling (Peyron et al., 1998). We conclude that MHb labeling by retrograde tracer in the present study is most likely a result of tracer injection spread into neighboring areas but a connectivity of some MHb neurons to monoaminergic nuclei cannot be completely ruled out.

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Collateralization is one of the principal mechanisms to provide the same information to several target nuclei. It is quite difficult, however, to estimate the number of multi-target neurons by tracing experiments. Each injection only covers a portion of each nucleus. Currently it is not possible to target all neurons of one nucleus with conventional tracing

techniques. Consequently, only a portion of neurons that project to the selected target nucleus are retrogradely labeled in the LHb. The same logic also applies to the double-labeled neurons stemming from dual retrograde injections which bares the possibility that the number of double labeled neurons may vary as a function of the size of the injected target areas. In our present investigation we cannot exclude this possibility. We believe, however, that areas covered by the injections are representative for the entire nucleus. We noticed no significant difference in LHb subnuclear distribution pattern of retrogradely labeled neurons in relation to different injection sites within a target nucleus (Fig. 3). In addition, our view is supported by the differences in LHb retrograde labeling strength from DR and MnR injections. There is convincing evidence that LHb efferent projection density to the MnR area is larger than to one to DR (Araki et al., 1988). This is reflected by our results, which show that the number of retrogradely labeled LHb neurons from MnR injections is 4.5 times higher from DR injections (Table 1). As a result the number of double labeled DR/VTA projecting neurons show a three-fold increase compared to MnR/VTA projecting doublelabeling in the LHb. However, the respective ratios of double-labeled neurons to all labeled neurons shows no difference between the two populations: The respective percentages for DR/VTA and MnR/VTA double-labeling are 1.2% and 1.3% of all labeled neurons. At the same time the number of retrogradely labeled neurons from VTA injections did not vary. This suggests that increases in raw counts of retrograde labeling from a single nucleus injection resulted in increases in number of double-labeled neurons while the respective ratio remains constant. Because a tracer injection can only cover a portion of entire nucleus, we feel that a proportional presentation reflects the distribution of projection neurons more accurately than raw neuronal count numbers.

Role of LHb in the modulation of monoaminergic circuits

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The LHb sends dense and direct projections to the majority of monoaminergic nuclei of the brainstem including VTA, DR, and MnR (Herkenham and Nauta, 1979). Several lines of evidence support the notion that increased LHb activity decreases monoaminergic activity in brainstem nuclei (Kalen et al., 1985; Christoph et al., 1986; Kalen et al., 1986; Geisler et al., 2003; Ji and Shepard, 2007; Brinschwitz et al., 2010). Electrical stimulations demonstrate that habenular inputs to monoaminergic cells are mostly inhibitory (Wang and Aghajanian, 1977; Ji and Shepard, 2007; Omelchenko et al., 2009; Brinschwitz et al., 2010). In constrast, LHb projection neurons mainly use glutamate as excitatory transmitter (Kalen et al., 1985; Kalen et al., 1986; Behzadi et al., 1990; Ferraro et al., 1996; Brinschwitz et al., 2010). Local GABAergic neurons within the monoamine nuclei often are the targets of the LHb efferents and thereby inhibit monoamine release in VTA (Omelchenko et al., 2009; Brinschwitz et al., 2010) and DR (Ferraro et al., 1996). However, there also is a portion of glutamatergic LHb neurons, which directly synapse onto dopaminergic and serotonergic neurons (Ferraro et al., 1996; Brinschwitz et al., 2010).

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In addition, there is another group of neurons in the LHb, which projects to the recently identified rostromedial tegmental nucleus (RMTg) that serves as a central GABAergic relay nucleus to provide inhibitory input to a variety of brainstem nuclei in response to excitatory LHb innervation (Jhou et al., 2009a; Jhou et al., 2009b; Kaufling et al., 2009). However, the LHb neurons projecting to the VTA are distinct from the ones targeting the RMTg (Li et al., 2011) indicating a functional difference between both pathways. Thus, RMTg neurons are mostly activated by aversive stimuli, such as foot shocks and food deprivation that result in passive fear behavior (Jhou et al., 2009a).

Many LHb neurons are spontaneously active (Kim and Chang, 2005; Weiss and Veh, 2011) and consequently exert tonic inhibition on monoamine nuclei even in the absence of afferent input (Kim and Chang, 2005; Lecourtier et al., 2008; Kim, 2009). Recently, our group has shown that LHb neurons projecting to DR, MnR, and VTA express mRNAs for all four

known HCN channel subunits (Poller et al., 2011). The results of our present study extend these findings by demonstrating that the vast majority of efferent LHb neurons target each of the three monoamine nuclei separately, permitting the LHb to independently modulate DR, MnR, and VTA activity.

Monoamine LHb afferent connections in a subnuclear context

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A massive input of LHb to major monoamine nuclei is well-known and documented (Herkenham and Nauta, 1979; Kalen et al., 1985; Behzadi et al., 1990). In contrast, it is not known, whether these neurons potentially are concentrated in one of the habenular subnuclei (Andres et al., 1999; Geisler et al., 2003). Here, we labeled, quantified, and assigned individual inputs to their respective habenular subnuclei. A comparison of electrophysiological and morphological characteristics of LHb neurons across subnuclei revealed no differences. Thus, every subnucleus is comparably equipped with a similar set of neurons displaying akin physiology (Weiss and Veh, 2011). In agreement with this is the observation that almost all LHb neurons express mRNA encoding for at least one hyperpolarization-activated cation (HCN) channel subunit (Poller et al., 2011). However, LHb subnuclear functional specificity has been demonstrated (Kowski et al., 2009). Whether all morphologically distinct LHb subnuclei represent functional correlates needs to be demonstrated.

The present study indicates a parallel construct of descending LHb fibers that arise from the same subnuclei and project to individual monoaminergic nuclei. The majority of these efferent neurons is provided by the subnuclei LHbMPc, LHbMC, and LHbLMc, which are medially to centrally located within the LHb. There is also a small population of habenular neurons that contained tracers from both injections, indicating that such neurons might be capable to simultaneously affect neurons in the VTA and DR or MnR. On the other hand, they simply may be remains of sharpening synapse specificity during development. Only 1 to 2%

of all labeled habenular cells embodied both tracers. Future studies will have to show electrophysiological evidence that such LHb neurons are capable of simultaneously provide input to two different monoaminergic nuclei.

All investigated efferent fibers descend through the fasciculus retroflexus. Consequently some tracer might have been taken up by fibers of passage, despite all precaution (see also methodological considerations). Interestingly, we often observed that local concentrations of tracer-containing neurons with the LHb which formed small heterogeneous clusters (Fig. 8). It could well be the case that these LHb projection neurons make dendritic contacts and thus communicate with each other. Whether such clusters or arrays of projection neurons possess network capabilities needs to be investigated.

LHb efferents to monoamine nuclei are part of a complex network geared for reward-related signal processing

The LHb is composed of a medial (LHbM), so-called limbic region, and a lateral (LHbL) "pallidal" part (for review see Geisler and Trimble, 2008). The LHbM receives afferents from limbic forebrain structures like the lateral hypothalamus and the lateral preoptic area (Kowski et al., 2008), whereas the LHbL receives supply predominately from the entopeduncular nucleus (Parent et al., 1981). In addition, the connectivity with every monoamine nucleus in the hindbrain is reciprocal (Herkenham and Nauta, 1977, 1979).

The LHb-VTA pathway has been extensively investigated in the context of reward-based decision making. Both structures assume opposing activity states in response to the comparison of expected and received reward (Matsumoto and Hikosaka, 2008; Bromberg-Martin et al., 2010a; Bromberg-Martin et al., 2010b). Adverse stimuli and chronic stress also can excite the LHb (Matsumoto and Hikosaka, 2008; Bromberg-Martin et al., 2010a; Bromberg-Martin et al., 2010b). Afferent inputs that mediate reward- and stress-related behaviors come from the globus pallidus (Hong and Hikosaka, 2008) or its rat homologous

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structure, the entopeduncular nucleus, from the lateral hypothalamus, or lateral preoptic area (Kowski et al., 2008). The neurochemistry and the subnuclear connectivity of these afferents is currently unknown. Preliminary data from our group suggests that projections from the lateral hypothalamus to the LHb are glutamatergic and synapse onto VTA projection neurons in the LHb (Poller et al., 2010). This suggests that LH signals can excite the LHb, which in turn largely inhibits VTA activity. Future studies will be aimed to identify the neurochemical identity of other LHb afferents. This will elucidate the mechanisms by which the LHb modulate monoaminergic circuits.

Dorsal raphe neurons are modulated by signals that encode for a particular reward size regardless whether the reward is predicted or not (Nakamura et al., 2008). In agreement, serotonin levels in the DR rise during extended waiting time for a reward (Miyazaki et al., 2011a). Serotonin concentrations in the DR also rise during the longer delay to obtain a larger award over a small immediate reward (Miyazaki et al., 2011b). In this context, the LHb most likely modulates DR activity during an anticipatory phase through the limbic input that the LHb receives.

The median raphe nucleus is also involved in reward signaling but its exact role or function is less clear. Administration of the GABA-B agonist baclofen into either raphe nuclei is rewarding (Wirtshafter et al., 1993). The reinforcing effect was larger in the DR, but self-administration of baclofen was much more readily acquired by injections into the MnR than into the DR (Shin and Ikemoto, 2010). One explanation for this observation may be that MnR has much stronger serontonergic projections to the hippocampus than the DR (van de Kar and Lorens, 1979). Therefore, the MnR may represent a region geared for reward-mediated learning. In addition, non-serotonergic neurons in the MnR facilitate mesolimbic dopamine neurotransmission (Paris and Lorens, 1987; Wirtshafter and McWilliams, 1987), demonstrating complexity and interactions between different monoaminergic systems in the context of reward processing. The LHb, by modulating both, dopamine and serotonin

providing nuclei, may act as their upstream modulator of anticipatory information that originates in the forebrain limbic system. The results from the present study imply that the LHb can separately transmit those inputs to each monoamine target nucleus and thereby fine-tune monoaminergic output to relevant forebrain regions. This may be of special importance for potentially novel treatments of mood disorders, for which the LHb is an excellent target (Sartorius et al., 2010; Li et al., 2011; Winter et al., 2011).

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References

Abercrombie M. 1946. Estimation of nuclear population from microtome sections. Anat Rec 94:239-247.

Andres KH, von During M, Veh RW. 1999. Subnuclear organization of the rat habenular complexes. J Comp Neurol 407:130-150.

Araki M, McGeer PL, Kimura H. 1988. The efferent projections of the rat lateral habenular nucleus revealed by the PHA-L anterograde tracing method. Br Res 441:319-330.

Behzadi G, Kalen P, Parvopassu F, Wiklund L. 1990. Afferents to the median raphe nucleus of the rat: retrograde cholera toxin and wheat germ conjugated horseradish peroxidase tracing, and selective D-[3H]aspartate labelling of possible excitatory amino acid inputs. Neuroscience 37:77-100.

Brinschwitz K, Dittgen A, Madai VI, Lommel R, Geisler S, Veh RW. 2010. Glutamatergic axons from the lateral habenula mainly terminate on GABAergic neurons of the ventral midbrain. Neuroscience 168:463-476.

Bromberg-Martin ES, Matsumoto M, Hikosaka O. 2010a. Distinct tonic and phasic anticipatory activity in lateral habenula and dopamine neurons. Neuron 67:144-155.

Bromberg-Martin ES, Matsumoto M, Hong S, Hikosaka O. 2010b. A pallidus-habenula-dopamine pathway signals inferred stimulus values. J Neurophysiol 104:1068-1076.

Chang HT, Kuo H, Whittaker JA, Cooper NGF. 1990. Light and electron microscopic analysis of projection neurons retgrogradely labeled with Fluoro-Gold: notes on the application of antibodies to Fluoro-Gold. J Neurosci Meth 35:31-37.

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

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Ellison G. 1994. Stimulant-induced psychosis, the dopamine theory of schizophrenia, and the habenula. Brain Res Brain Res Rev 19:223-239.

Ferraro G, Montalbano ME, Sardo P, La Grutta V. 1996. Lateral habenular influence on dorsal raphe neurons. Brain Res Bull 41:47-52.

Geisler S, Trimble M. 2008. The lateral habenula: no longer neglected. CNS Spectr 13:484-489.

Geisler S, Heilmann H, Veh RW. 2002. An optimized method for simultaneous demonstration of neurons and myelinated fiber tracts for delineation of individual trunco- and palliothalamic nuclei in the mammalian brain. Histochem Cell Biol 117:69-79.

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

Herkenham M, Nauta WJ. 1977. 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 173:123-146.

Herkenham M, Nauta WJ. 1979. Efferent connections of the habenular nuclei in the rat. J Comp Neurol 187:19-47.

Hikosaka O. 2010. The habenula: from stress evasion to value-based decision-making. Nat Rev Neurosci 11:503-513.

Hong S, Hikosaka O. 2008. The globus pallidus sends reward-related signals to the lateral habenula. Neuron 60:720-729.

Jhou TC, Fields HL, Baxter MG, Saper CB, Holland PC. 2009a. The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. Neuron 61:786-800.

Jhou TC, Geisler S, Marinelli M, Degarmo BA, Zahm DS. 2009b. The mesopontine rostromedial tegmental nucleus: A structure targeted by the lateral habenula that

projects to the ventral tegmental area of Tsai and substantia nigra compacta. J Comp Neurol 513:566-596.

Ji H, Shepard PD. 2007. Lateral habenula stimulation inhibits rat midbrain dopamine neurons through a GABA(A) receptor-mediated mechanism. J Neurosci 27:6923-6930.

Kalen P, Karlson M, Wiklund L. 1985. Possible excitatory amino acid afferents to nucleus raphe dorsalis of the rat investigated with retrograde wheat germ agglutinin and D-[3H]aspartate tracing. Brain Res 360:285-297.

Kalen P, Pritzel M, Nieoullon A, Wiklund L. 1986. Further evidence for excitatory amino acid transmission in the lateral habenular projection to the rostral raphe nuclei: lesion-induced decrease of high affinity glutamate uptake. Neurosci Lett 68:35-40.

Kaufling J, Veinante P, Pawlowski SA, Freund-Mercier MJ, Barrot M. 2009. Afferents to the GABAergic tail of the ventral tegmental area in the rat. J Comp Neurol 513:597-621.

Kim U. 2009. Topographic commissural and descending projections of the habenula in the rat. J Comp Neurol 513:173-187.

Kim U, Chang SY. 2005. Dendritic morphology, local circuitry, and intrinsic electrophysiology of neurons in the rat medial and lateral habenular nuclei of the epithalamus. J Comp Neurol 483:236-250.

Klemm WR. 2004. Habenular and interpeduncularis nuclei: shared components in multiple-function networks. Med Sci Monit 10: RA261-73

Kowski AB, Veh RW, Weiss T. 2009. Dopaminergic activation excites rat lateral habenular neurons in vivo. Neuroscience 161:1154-1165.

Kowski AB, Geisler S, Krauss M, Veh RW. 2008. Differential projections from subfields in the lateral preoptic area to the lateral habenular complex of the rat. J Comp Neurol 507:1465-1478.

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Lecourtier L, Kelly PH. 2007. A conductor hidden in the orchestra? Role of the habenular complex in monoamine transmission and cognition. Neurosci Biobehav Rev 31:658-672.

Lecourtier L, Neijt HC, Kelly PH. 2004. Habenula lesions cause impaired cognitive performance in rats: implications for schizophrenia. Eur J Neurosci 19:2551-2560.

Lecourtier L, Defrancesco A, Moghaddam B. 2008. Differential tonic influence of lateral habenula on prefrontal cortex and nucleus accumbens dopamine release. Eur J Neurosci 27:1755-1762.

Li B, Piriz J, Mirrione M, Chung C, Proulx CD, Schulz D, Henn F, Malinow R. 2011. Synaptic potentiation onto habenula neurons in the learned helplessness model of depression. Nature 470:535-539.

Marcinkiewicz, M, Morcos, R, Chretien, M. 1989. CNS connections with the median raphe nucleus: retrograde tracing with WGA-apoHRP-Gold complex in the rat. J Comp Neurol 289:11-35

Matsumoto M, Hikosaka O. 2007. Lateral habenula as a source of negative reward signals in dopamine neurons. Nature 447:1111-1115.

Matsumoto M, Hikosaka O. 2008. Negative motivational control of saccadic eye movement by the lateral habenula. Prog Brain Res 171:399-402.

Miyazaki K, Miyazaki KW, Doya K. 2011a. Activation of dorsal raphe serotonin neurons underlies waiting for delayed rewards. J Neurosci 31:469-479.

Miyazaki KW, Miyazaki K, Doya K. 2011b. Activation of the central serotonergic system in response to delayed but not omitted rewards. Eur J Neurosci 33:153-160.

Nakamura K, Matsumoto M, Hikosaka O. 2008. Reward-dependent modulation of neuronal activity in the primate dorsal raphe nucleus. J Neurosci 28:5331-5343.

Omelchenko N, Bell R, Sesack SR. 2009. Lateral habenula projections to dopamine and GABA neurons in the rat ventral tegmental area. Eur J Neurosci 30:1239-1250.

Parent A, Gravel S, Boucher R. 1981. The origin of forebrain afferents to the habenula in rat, cat and monkey. Brain Res Bull 6:23-38.

Paris JM, Lorens SA. 1987. Intra-median raphe infusions of muscimol and the substance P analogue DiMe-C7 produce hyperactivity: role of serotonin neurons. Behav Brain Res 26:139-151.

Paxinos G, Watson C. 1998. The Rat Brain in Stereotaxic Coordinates, 4th ed. Edition. New York: Academic Pres.

Peyron C, Petit JM, Rampon C, Jouvet M, Luppi PH. 1998. Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. Neuroscience 82:443-68.

Poller WC, Bernard R, Derst C, Weiss T, Madai VI, Veh RW. 2011. Lateral habenular neurons projecting to reward-processing monoaminergic nuclei express hyperpolarization-activated cation (HCN) channels. Neuroscience: 193:205-216~~in press~~.

Poller WC, Bernard R, Madai VI, Kahl T, Laube G, Veh RW. 2010. Identification of lateral habenular neurons relaying hypothalamic input to monoaminergic hindbrain circuits. Soc Neurosci Abstr:A 511.522.

Sandyk R. 1992. Pineal and habenula calcification in schizophrenia. Int J Neurosci 67:19-30.

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

Shepard PD, Holcomb HH, Gold JM. 2006. Schizophrenia in translation: the presence of absence: habenular regulation of dopamine neurons and the encoding of negative outcomes. Schizophr Bull 32:417-421.

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Shin R, Ikemoto S. 2010. The GABA(B) receptor agonist baclofen administered into the median and dorsal raphe nuclei is rewarding as shown by intracranial self-administration and conditioned place preference in rats. Psychopharmacology (Berl).

Stern WC, Johnson A, Bronzino JD, Morgane PJ. 1979. Effects of electrical stimulation of the lateral habenula on single-unit activity of raphe neurons. Exp Neurol 65:326-342.

Sutherland, RJ. 1982. The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. Neurosci Biobehav Rev 6:1-13.

Ullsperger M, von Cramon DY. 2003. Error monitoring using external feedback: specific roles of the habenular complex, the reward system, and the cingulate motor area revealed by functional magnetic resonance imaging. J Neurosci 23:4308-4314.

van de Kar LD, Lorens SA. 1979. Differential serotonergic innervation of individual hypothalamic nuclei and other forebrain regions by the dorsal and median midbrain raphe nuclei. Brain Res 162:45-54.

Wang RY, Aghajanian GK. 1977. Physiological evidence for habenula as major link between forebrain and midbrain raphe. Science 197:89-91.

Weiss T, Veh RW. 2011. Morphological and electrophysiological characteristics of neurons within identified subnuclei of the lateral habenula in rat brain slices. Neuroscience 172:74-93.

Winter C, Vollmayr B, Djodari-Irani A, Klein J, Sartorius A. 2011. Pharmacological inhibition of the lateral habenula improves depressive-like behavior in an animal model of treatment resistant depression. Behav Brain Res 216:463-465.

Wirtshafter D, McWilliams C. 1987. Suppression of locomotor activity produced by acute injections of kainic acid into the median raphe nucleus. Brain Res 408:349-352.

Wirtshafter D, Stratford TR, Pitzer MR. 1993. Studies on the behavioral activation produced by stimulation of GABAB receptors in the median raphe nucleus. Behav Brain Res 59:83-93

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List of abbreviations

DR	dorsal raphe nucleus
IPN	interpeduncular nucleus
lfp	longitudinal fasciculus of the pons
LH	lateral hypothalamic area
LHb	lateral habenular complex
LHbL	lateral division of the lateral habenular complex
LHbLB	basal part of the lateral division of the lateral habenular complex
LHbLMc	magnocellular part of the lateral division of the lateral habenular complex
LHbLMg	marginal part of the lateral division of the lateral habenular complex
LHbLO	oval part of the lateral division of the lateral habenular complex
LHbLPc	parvocellular part of the lateral division of the lateral habenular complex
LHbM	medial division of the lateral habenular complex
LHbMA	anterior part of the medial division of the lateral habenular complex
LHbMC	central part of the medial division of the lateral habenular complex
LHbMMg	marginal part of the medial division of the lateral habenular complex
LHbMPc	parvocellular part of the medial division of the lateral habenular complex
LHbMS	superior part of the medial division of the lateral habenular complex
MHb	medial habenular nucleus
ml	medial lemniscus
MnR	median raphe nucleus
Pn	pons
PnO	pontine reticular nucleus, oral part
RMTg	rostromedial tegmental nucleus
RNu	red nucleus

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VLPAG	ventrolateral periaqueductual grey
VTA	ventral tegmental area

Figure legends

Figure 1. Schematic representation of the two models presenting the efferent connectivity of the LHb to the monoaminergic midbrain nuclei, DR, MnR, and VTA, as investigated in by the present study. Each LHb neuron may separately target only one of the three monoamine nuclei (Model A). Alternatively, individual LHb projection neurons may target two (DR and VTA or MnR and VTA) or more monaminergic nuclei through axon collaterals (Model B).

Figure 2. Adjacent series of Klüver-Barrera stained sections display habenular cytoarchitecture (A-F), partially employed for identification of LHb subnuclei. Graphical representation of retrogradely labeled neurons after concomitant delivery of WGA-poHRP-gold (blue dots) and fluorogold (magenta) as tracers into VTA and DR, respectively (A'-F'). Scale bar equals 250 µm. See list for abbreviations.

Figure 3—Injection sites illustrations. Retrograde tracer injection sites as shown as hatched areas in coronal section illustrations. The left panel (A-C) displays all VTA injections whereas the right panel marks DR and MnR (D-F) injection sites. Outlines of the target nuclei as shown as dashed areas. The respective animal is assigned to each injection. Approximated Bregma levels are shown below each illustration.

Figure 4. Summary graphs displaying subnuclear distribution of retrogradely labeled habenular neurons resulting from microinjections into DR (A), MnR (B), and VTA (C). Averages of neuronal counts are shown as portion of all retrogradely labeled neurons stemming from one injection. (D). Anterior to posterior distribution of habenular retrogradely labeled neurons after tracer injection in DR, MnR, or VTA. In coronal serial brain sections

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traced habenular neurons were evaluated every 250 µm. Approximated Bregma (Br) levels for anterior and posterior ends of the LHb are displayed. See list for abbreviations.

Figure 5. Adjacent series with Kluever-Barrera stained sections display habenular cytoarchitecture (A-F) partially employed for identification of LHb subnuclei. Graphical representation of retrogradely labeled neurons after concomitant delivery of WGAapoHRP-Au tracer and flurogold tracer into VTA and MnR, respectively (A'-F'). Magenta dots serve to illustrate labeled neurons resulting from MnR tracer injection, blue black dots represent neurons labeled retrogradely by VTA tracer injection. Scale bar equals 250 µm. See list for abbreviations.

Figure 6. – Representative images from different LHb neurons that contain black tracer particles from a VTA injection with WGApoHRP-Au and brown-colored DAB-immunolabeled containing fluorogold tracer particles from MnR or DR microinjection. Black arrows indicate cells that contain both tracers.

Figure 7. Retrograde tracing results from control injections. Retrograde tracer was microinjectioned into the ventrolateral periaqueductulal grey ~~red nucleus~~ (A) and ~~ventrolateral periaqueductulal grey~~ (C) red nucleus and did not produce significant retrograde labeling in the habenula (C and D). Tracer application into the paramedian raphe/pontine reticular nucleus, oral part (E) produced retrograde labeling predominantly in the lateral part of the LHb (F). Scale bar in A equals 1 mm and also applies for C and E. Scale bar in B equals 250 µm and also applies for D and F. See list for abbreviations.

Figure 8. (A) Image of coronal habenular section showing a cluster of DAB-immunolabeled neurons in the LHb, resulting from a FG-tracer injection in the MnR. (B) Enlargement of the

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cluster of retrogradely labeled LHb neurons taken from the black rectangular frame in A. Scale bar in A equals 250 µm, in B it equals 30 µm.

Table 1— Average raw counts of retrogradely labeled neurons in the habenular subnuclei after injections into DR, MnR, or VTA. Columns on the right represent average numbers of double labeled neurons in habenular subnuclei after DR-VTA dual injections and MnR-VTA dual injections.

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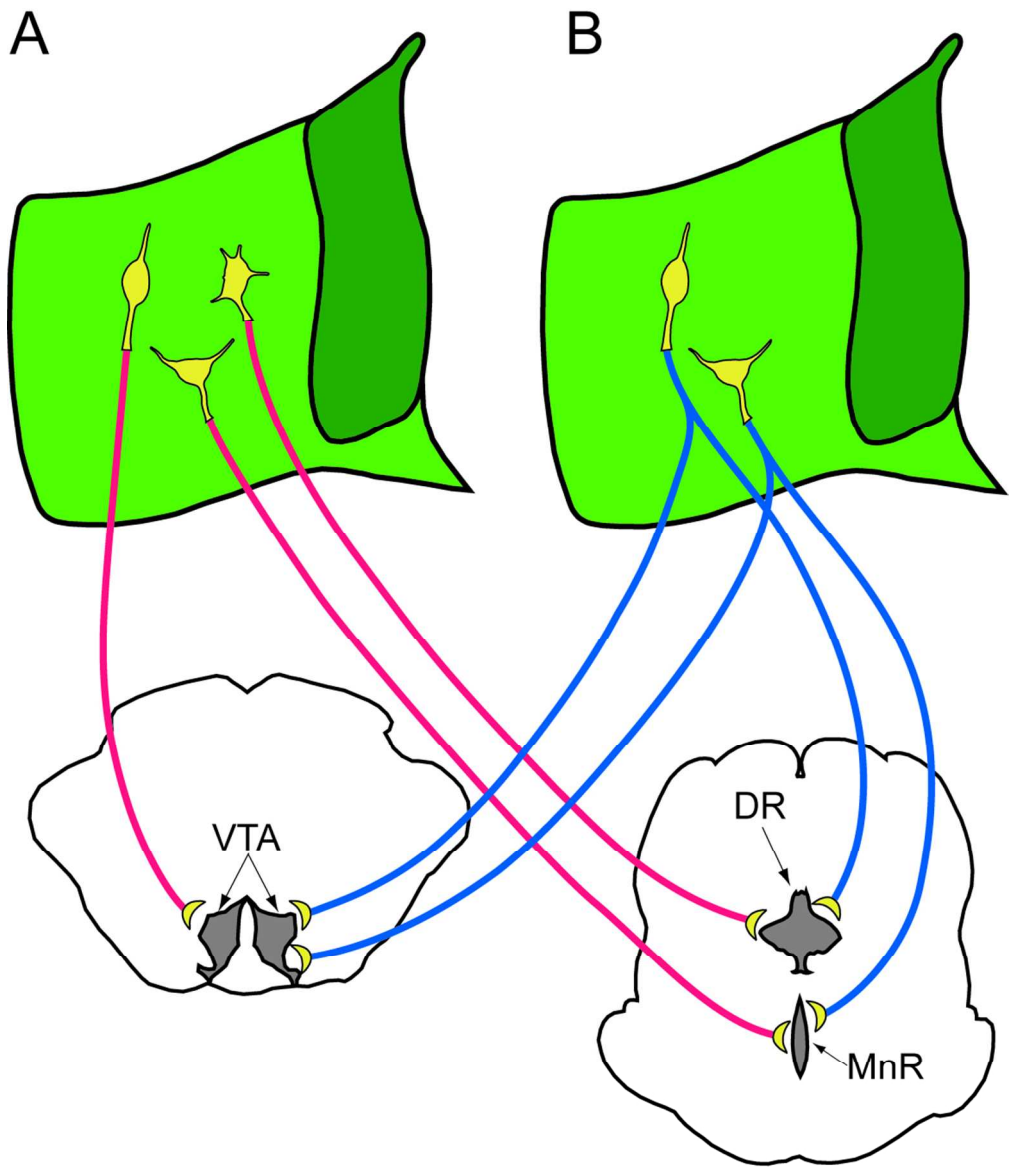

Schematic representation of the two models presenting the efferent connectivity of the LHb to the monoaminergic midbrain nuclei, DR, MnR, and VTA, as investigated in by the present study. Each LHb neuron may separately target only one of the three monoamine nuclei (Model A). Alternatively, individual LHb projection neurons may target two (DR and VTA or MnR and VTA) or more monaminergic nuclei through axon collaterals (Model B).
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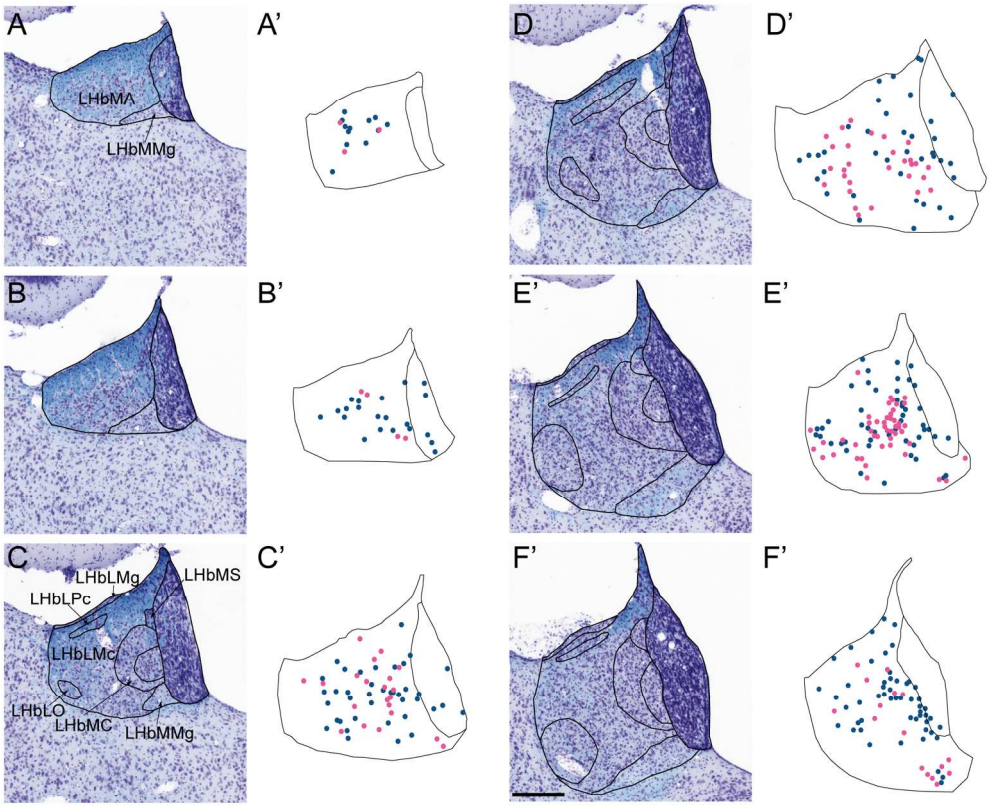

Adjacent series of Klüver-Barrera stained sections display habenular cytoarchitecture (A-F), partially employed for identification of LHb subnuclei. Graphical representation of retrogradely labeled neurons after concomitant delivery of WGA-poHRP-gold (blue dots) and fluorogold (magenta) as tracers into VTA and DR, respectively (A'-F'). Scale bar equals 250 µm. See list for abbreviations.
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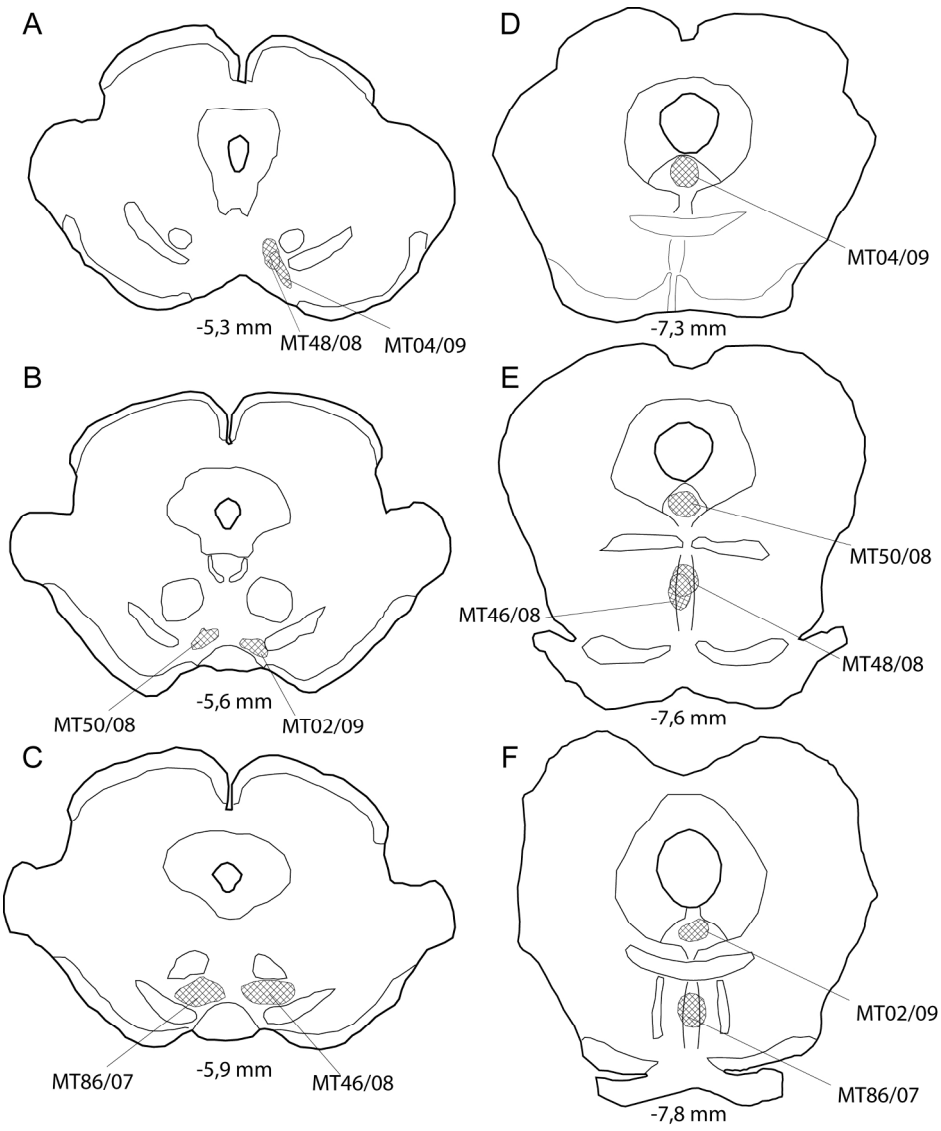

Injection sites illustrations. Retrograde tracer injection sites as shown as hatched areas in coronal section illustrations. The left panel (A-C) displays all VTA injections whereas the right panel marks DR and MnR (D-F) injection sites. Outlines of the target nuclei as shown as dashed areas. The respective animal is assigned to each injection. Approximated Bregma levels are shown below each illustration.
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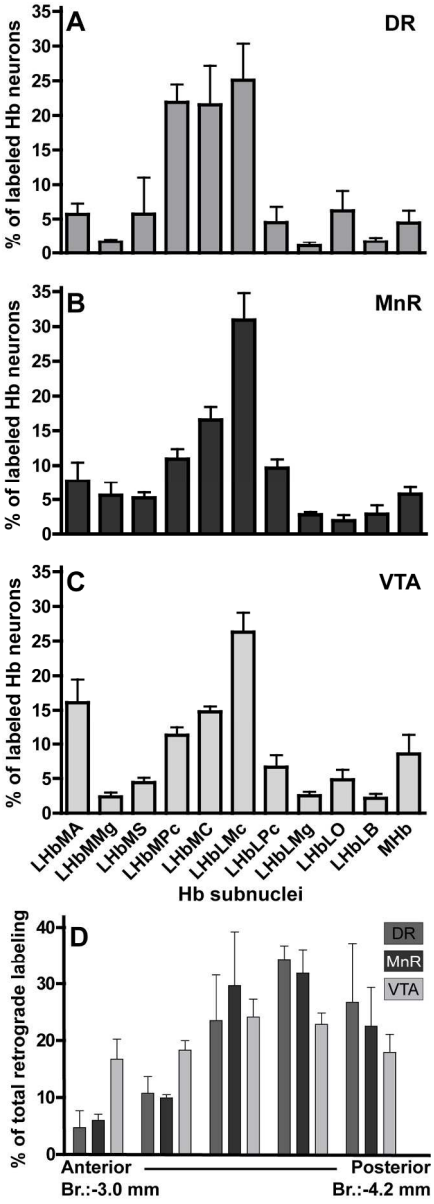

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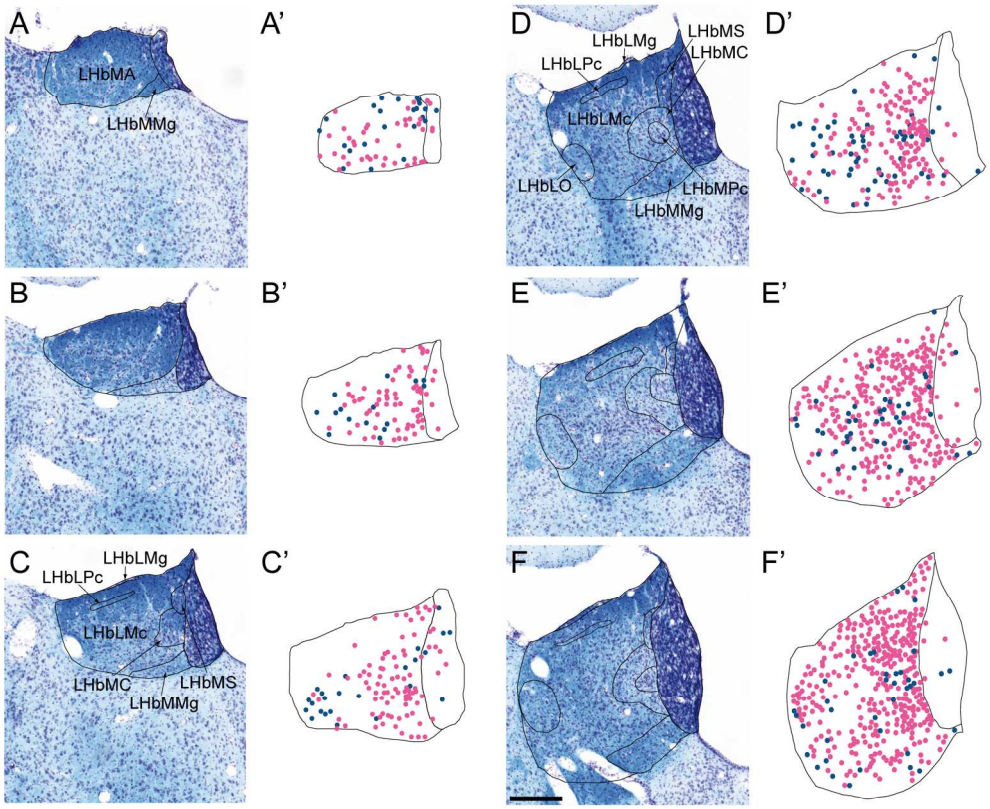

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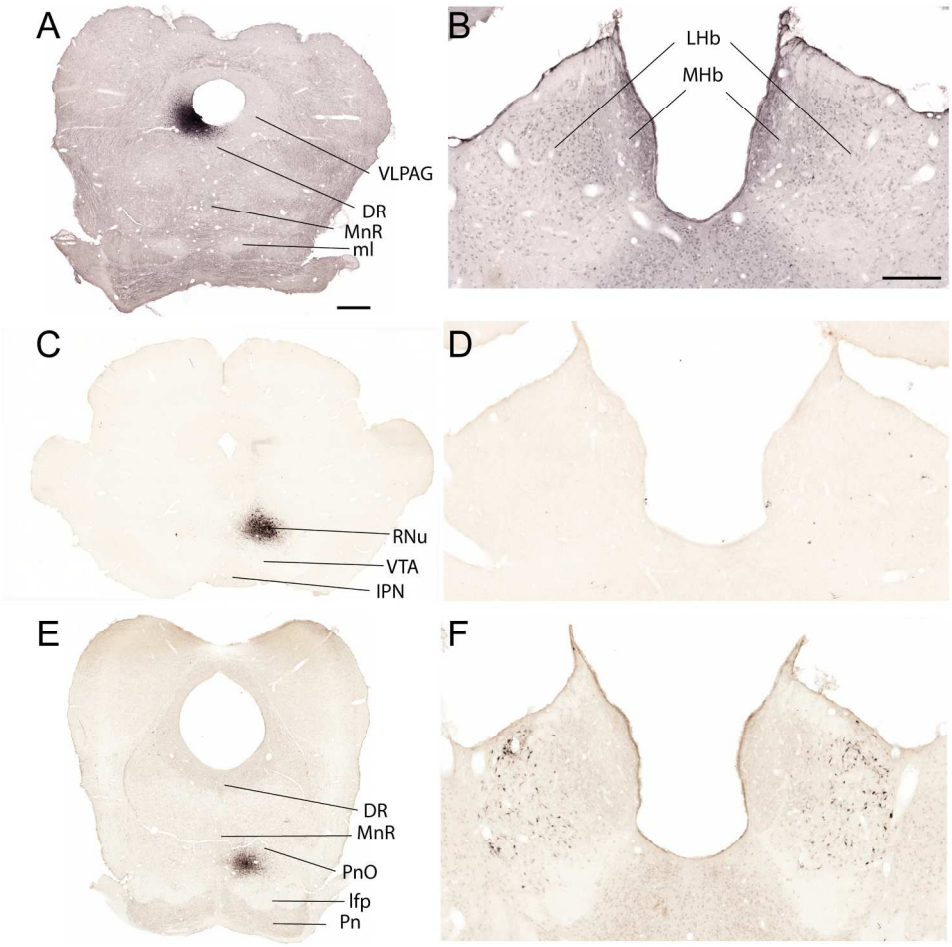

Retrograde tracing results from control injections. Retrograde tracer was microinjectioned into the ventrolateral periaqueductulal grey (A) and (C) red nucleus and did not produce significant retrograde labeling in the habenula (C and D). Tracer application into the paramedian raphe/pontine reticular nucleus, oral part (E) produced retrograde labeling predominantly in the lateral part of the LHb (F). Scale bar in A equals 1 mm and also applies for C and E. Scale bar in B equals 250 μm and also applies for D and F. See list for abbreviations.
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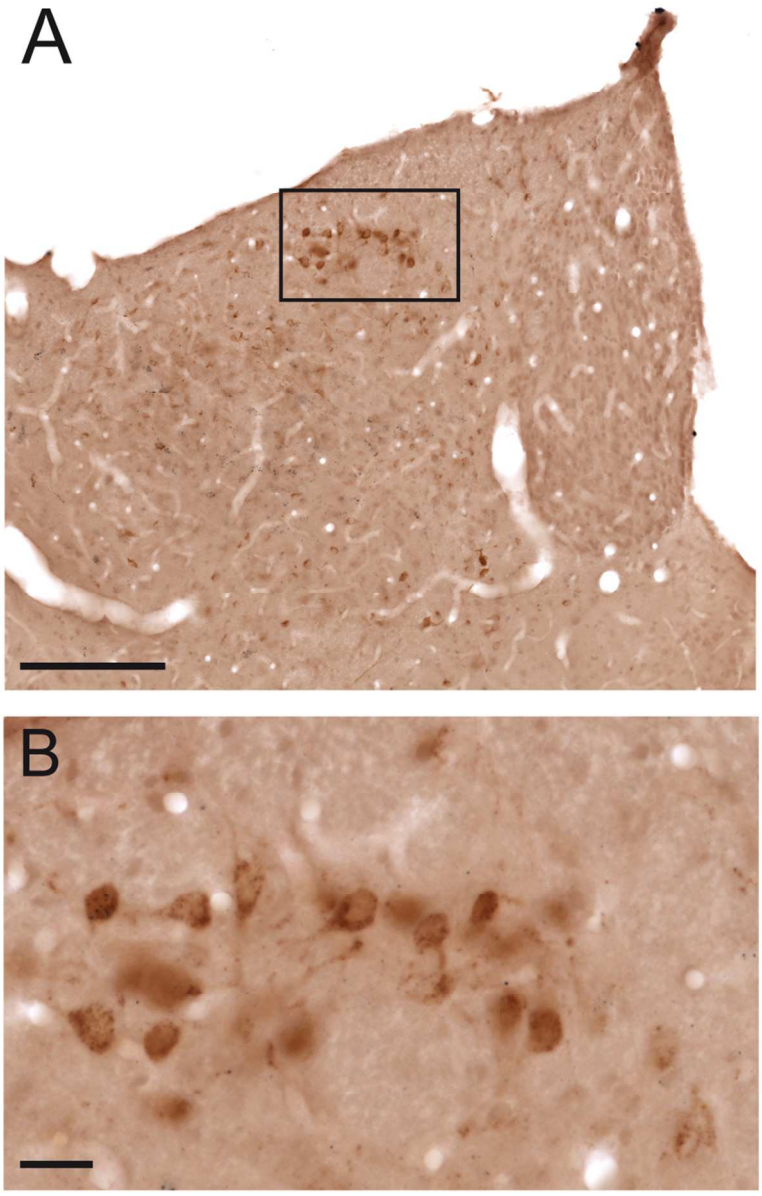

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96x116mm (300 x 300 DPI)

	DR (counts)	MR (counts)	VTA (counts)	DR-VTA	MR-VTA
LhbMA	14	88	60	1	3
LhbMMg	5	54	9	0	1
LHbMS	22	62	11	0	0
LHbMPc	53	129	34	0	4
LHbMC	49	181	52	2	4
LHbLB	3	38	6	0	0
LHbLPc	15	101	19	0	1
LHbLMg	3	29	8	0	0
LHbLO	9	27	18	0	1
LHbLMc	49	316	86	1	4
MHb	11	64	27	0	0
TOTAL	234	1088	330	6	18