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

DISSERTATION

Glutamatergic axons from the lateral habenula mainly terminate on GABAergic neurons of the ventral midbrain

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

Vorgelegt der Medizinischen Fakultät Charité –
Universitätsmedizin Berlin

von

Katja Brinschwitz
Geboren in Luckenwalde

Gutachter:

1.: Prof. R.W. Veh

2.: Priv.-Doz. Dr. med. Chr. W. Zimmermann

3.: Ph.D. P. Shepard

Datum der Promotion: 08.04.2011

Abstract (deutsch)

Einleitung: Dopaminerge Neurone in der ventralen tegmentalen Area (VTA) modulieren die neuronale Aktivität der Basalganglienschleifen. Die Ausschüttung von Dopamin in den Zielarealen wie den Nucleus accumbens führt dabei zu positiven Emotionen, die in ihrer Summe als Belohnung wahrgenommen werden. Im Gegenzug führt eine Hemmung der Dopaminsekretion zu negativen Emotionen (negative reward). Die VTA ist somit unabdingbar für Lernprozesse und daraus resultierendes, zielgerichtetes Handeln. Die Neurone der VTA integrieren dafür stimulierende und inhibitorische Einflüsse aus verschiedenen kortikalen und subkortikalen Zentren. Ein wichtiger Zufluss von Informationen erwächst der VTA aus dem lateralen Habenularkomplex (LHb). In früheren Studien konnte bereits gezeigt werden, dass eine elektrische Stimulation von Neuronen in der LHb zu einer Hemmung der Dopaminausschüttung in der VTA führt. Die Charakterisierung dieser Projektionen wurde bisher nur unzureichend untersucht. Insbesondere die beteiligten Neurotransmitter und die Zielneurone der in der VTA waren nicht bekannt. Ziel der vorliegenden Arbeit war es, durch eine Kombination verschiedener anatomischer Techniken eine genaue Charakterisierung der Projektionen aus der LHb und der angesteuerten Zielneurone in der VTA zu erreichen.

Resultate: Die Existenz von Projektionen aus der LHb in die VTA wurde durch verschiedenen Tracingtechniken nachgewiesen. Dabei führte die Injektion von Tetramethylrodamin-markiertem Dextranamin (TMR-DA) in die LHb zu einer Anreicherung des Tracers in Axonterminalen in der VTA (anterogrades Tracing). Im Gegenzug konnte nach Injektion von Gold-markiertem Weizenkeim Agglutinin (ApoHRP-WGA-gold) in die VTA ein retrograder Transport der Substanz in die Neuronen der LHb dargestellt werden (retrogrades Tracing). Durch nicht radioaktive In-Situ-Hybridisierung wurde dann gezeigt, dass die Mehrzahl der WGA-markierten Neurone in der LHb mRNA für den vesikulären Glutamattransporter vGluT2 exprimieren. Dieser Befund wurde durch den immuncytochemischen Nachweis von vGluT2 in TMR-DA-positiven Axonterminalen in der VTA ergänzt. GAD-immunreaktive GABAerge Axonterminalen in der VTA enthielten dagegen keine Tracersubstanz. Die Ursprungsneurone für die Projektionen aus der LHb in die VTA konnten damit als exzitatorische, glutamaterge Neurone identifiziert werden.

Die Charakterisierung der Zielzellen erfolgte mittels Pre- und Postembedding-techniken in der Elektronenmikroskopie. Dazu wurden dopaminerge Zellen mit Antikörpern gegen Tyrosinhydroxylase, GABAerge Zellen mit Antikörpern gegen GABA und GAD, sowie glutamaterge Neurone mit Antikörpern gegen vesikuläre Glutamattransporter (vGluT1 und vGluT2) markiert. TMR-DA-positive Axonterminalen, mittels DAB visualisiert, fanden sich dabei hauptsächlich an GABAergen Dendriten, während direkte Projektionen auf dopaminerge Neurone selten waren.

Schlussfolgerung: In der vorliegenden Arbeit konnte gezeigt werden, dass exzitatorische, glutamaterge Neurone der LHb direkt auf GABAerge Interneurone in der VTA projizieren. Diese Projektionen bilden das morphologische Korrelat für die hemmende Wirkung der LHb auf die Dopaminfreisetzung aus den Neuronen der VTA und ihren Axonterminalen. Die neuen Erkenntnisse verbessern unser Verständnis der Funktionsweise der VTA und können so vielleicht zur Entwicklung neuer Behandlungsstrategien für Erkrankungen wie Depression und Schizophrenie beitragen.

Schlagwörter: VTA, laterale Habenula, Dopamin, Schizophrenie

Abstract (English)

The concept of cortical-subcortical loops emphasizes the importance of the basal ganglia for motor, psychomotor, and emotional cortical functions. These loops are bidirectionally controlled by the midbrain dopaminergic system, predominantly but not exclusively at the level of the striatum, including the accumbens nucleus. Successful behaviors increase the activities of the mesostriatal (arising in the complex part of the substantia nigra) and mesolimbic (arising in the ventral tegmental area, VTA) neurons, thereby reinforcing the corresponding actions. In contrast, unsuccessful behaviors result in an increased activation of the lateral habenular complex (LHb), thereby decreasing the activities of mesolimbic neurons. Correspondingly, electrical stimulation of the LHb effectively blocks neuronal activity in the VTA. Whether this block is due to an inhibitory projection from the LHb to the VTA, or whether axons from excitatory LHb neurons target inhibitory neurons within the VTA, is currently not known.

Here we show, using in-situ-hybridization and immunocytochemical double labelling at the light and electron microscopic level, that GABAergic neurons are scarce in the LHb and that glutamatergic axons from the LHb mostly target GABAergic neurons in the VTA and the mesopontine rostromedial tegmental nucleus. These data explain the inhibitory effect of LHb activation on the VTA. In addition, however, a small number of LHb terminals in the VTA actually contacts dopaminergic neurons. The biological importance of these terminals requires further investigation.

Key words: reward; mesolimbic dopamine; nucleus accumbens; connections; error detection; electron microscopy.

ABBREVIATIONS

Acb: accumbens nucleus

AMPA: alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

ApoHRP-WGA: apo-horseradish peroxidase-wheat germ agglutinin

CLi: caudal linear nucleus of raphe

cDNAs: complementary DNA

CNS: central nerve system

CtB: cholera toxoid

cp: central peduncle

DA: dopamine

DIG: digoxigenin

EM: electron microscopy

EP: entopeduncular nucleus

fr: fasciculus retroflexus

fMRI: magnetic functional resonance imaging

GABA: gamma amino butyric acid

GAD65: glutamate acid decarboxylase (65 kD)

GAD67: glutamate acid decarboxylase (67 kD)

Gpi: globus pallidus internal segment

IF: interfascicular nucleus

IP: interpeducular nucleus

IgG: immunoglobulin G

LHb: lateral habenular complex

LHbLB: basal subnucleus of the LHb

LHbLO: oval subnucleus of the LHb

LHbMC: central subnucleus of the LHb

LHbLMc: magnocellular subnucleus of the lateral division of the LHb

LHbMA: anterior subnucleus of the medial division of the LHb

LHbMMg: marginal subnucleus of the medial division of the LHb

LHbLMg: marginal subnucleus of the lateral division of the LHb

LHbMS: superior subnucleus of the medial division of the LHb

LHbMPc: parvocellular subnucleus of the medial division of the LHb

LHbLPc: parvocellular subnucleus of the lateral division of the LHb

LC: central lineal nucleus

LM: light microscopy

LR: rostral lineal nucleus

MbPhaL: Phaseolus vulgaris-leucoagglutinin

MHc: medial habenular complex

ml: medial lemniscus

mp: mammillary peduncle

PAG: periaqueductal gray

PaR: pararubral nucleus

PBN: parabrachial pigmented nucleus

PCR: polymerase chain reaction

PN: paranigral nucleus

RLi: rostral lineal nucleus of raphe

RMTg: rostromedial tegmental nucleus

RN: red nucleus

RNAse: ribonuclease

RT: room temperature

SN: substantia nigra

SNc: substantia nigra pars compacta

SNr: substantia nigra pars reticularis

TH: tyrosine hydroxylase

TMR-DA: tetramethylrhodamine-conjugated dextran amine

tVTA: tail of the VTA

vGluT1: vesicular glutamate transporter 1

vGluT2: vesicular glutamate transporter 2

vGluT3: vesicular glutamate transporter 3

VTA: ventral tegmental area

3n: third nerve

Table of contents

3. Results

1. Introduction

Dopaminergic neurons in the ventral tegmental area (VTA) modulate basal ganglia circuits and regulate motor and reward associated functions like food intake and reproduction. The release of dopamine in the target areas depends on different excitatory as well as inhibitory afferences to the VTA. Recent studies suggest that the lateral habenula (LHb) is an important modulator of midbrain DA neurons and exerts negative rewarding influence (Christoph et al. 1986; Matsumoto and Hikosaka 2007; Ji and Shepard 2007). A dysfunction of communication between the LHb and the VTA has been linked to the development of schizophrenia and depression (Winter et al. 2010; Caldecott-Hazard et al. 1988; Sartorius and Henn 2007; Morris et al. 1999). A comprehensive knowledge of the morphological and functional characteristics of the communication between the LHb and the VTA is therefore essential for the understanding of the pathophysiology of these debilitating diseases and may contribute to the development of novel therapeutic strategies.

1.1. The mesencephalic dopaminergic system plays a central role in the regulation of motor and emotional functions

The ventral tegmental area (VTA) and the substantia nigra (SN) are located in the ventro-medial region of the midbrain and contain the largest accumulation of dopaminergic neurons in the brain. Based on morphological criteria the VTA can be divided into six subnuclei (Fig. 1): the parabrachial pigmented nucleus (PBN), the paranigral nucleus (PN), the central linear nucleus (LC), the interfascicular nucleus (IF), and the rostral linear nucleus (LR) (Phillipson 1979; Halliday and Törk 1986; Oades and Halliday 1987). According to a recent study, the mesopontine rostromedial tegmental nucleus (RMTg) is also included into the VTA. Based on its location in the posterior part of the VTA it has been designated as tail of the VTA (tVTA) (Kauling et al. 2009).

The VTA contains two major neuronal populations which differ with regard to their neurotransmitter and enzyme content. The majority of the neurons in the VTA uses dopamine as neurotransmitter. These neurons are identified by their expression of tyrosine hydroxylase (TH) (Bayer and Pickel 1990). The second population produces the inhibitory neurotransmitter gamma amino butyric acid (GABA) and is characterized by the

expression of L-glutamic acid decarboxylase (GAD) (Mugnaini and Oertel 1985; Johnson and North 1992, Bayer and Pickel 1991). The tVTA mostly contains GABAergic neurons with low density of dopaminergic cells. In general both neuron populations form a network and act synergistically to control dopamine outflow from the VTA and the tVTA (Chen and Rice 2002). In this network, GABAergic neurons can act as interneurons which exert a direct inhibitory or indirect disinhibitory control on the dopaminergic neurons and influence dopaminergic outflow from the VTA (Johnson et al. 1992; Steffensen et al. 1998). Alternatively, the GABAergic cells project to the nucleus accumbens as well as to the prefrontal cortex (Carr and Sesack 2000; van Bocksteale and Pickel 1995).

In agreement with their diverse biological functions, VTA neurons project to a wide variety of cortical and subcortical structures. Important projections from the VTA terminate in the ventral striatum and the nucleus accumbens. In addition, VTA axons terminate on neurons in the prefrontal and insular cortex and in subcortical structures like the amygdala, the hypothalamic nuclei, the lateral habenula, the brainstem reticular formation and the monoaminergic nuclei (Carr and Sesack 2000; van Bocksteale and Pickel 1995; Oades and Halliday 1987; Swanson 1982).

1.2. Sources of excitatory, glutamatergic and cholinergic projections to dopaminergic neurons are widespread

Dopaminergic neurons in the VTA discharge spontaneously in a slow irregular pattern or in a bursting mode (Bunney and Aghajanian 1976; Gariano and Groves 1988). The resting activity can be modulated by excitatory and inhibitory afferences to the VTA. An enhanced neural activity correlates with an increased dopamine release in the target structures (Gonon 1988; Redgrave et al. 1999; Garris and Wightman 1994; Garris et al. 1994).

Sources of excitatory glutamatergic and cholinergic projections to the VTA include the laterodorsal tegmental nucleus, the prefrontal cortex, the lateral septum, the medial septum-diagonal band complex, the accumbens shell, the ventral pallidum, the medial and lateral preoptic area, the lateral hypothalamic area, the dorsal raphe, the periaqueductal

gray, and the mesencephalic and brainstem reticular formation (Christie et al. 1986; Sesack and Pickel 1992; Geisler and Zahm 2005; Geisler et al. 2007; Forster and Blaha 2000; Lodge and Grace 2006). However knowledge regarding the inhibitory afferences to the VTA is limited. Tonically active GABAergic projections originate from the ventral striatum (Wu et al. 1996; Zahm and Heimer 1990). In addition, there is cumulating functional and pharmacological evidence that the lateral habenula (LHb) takes part in the inhibitory control of the VTA (Ji and Shepard 2007; Ullsperger and von Cramon 2003; Shepard et al. 2006; Matsumoto and Hikosaka 2007). Supporting information dates back to observations that lesions of the habenular complexes result in an activation (Lisoprawski et al. 1980; Nishikawa et al. 1986) and that electrical stimulation of the LHb provokes an inhibition (Christoph et al. 1986) of mesencephalic DA neurons.

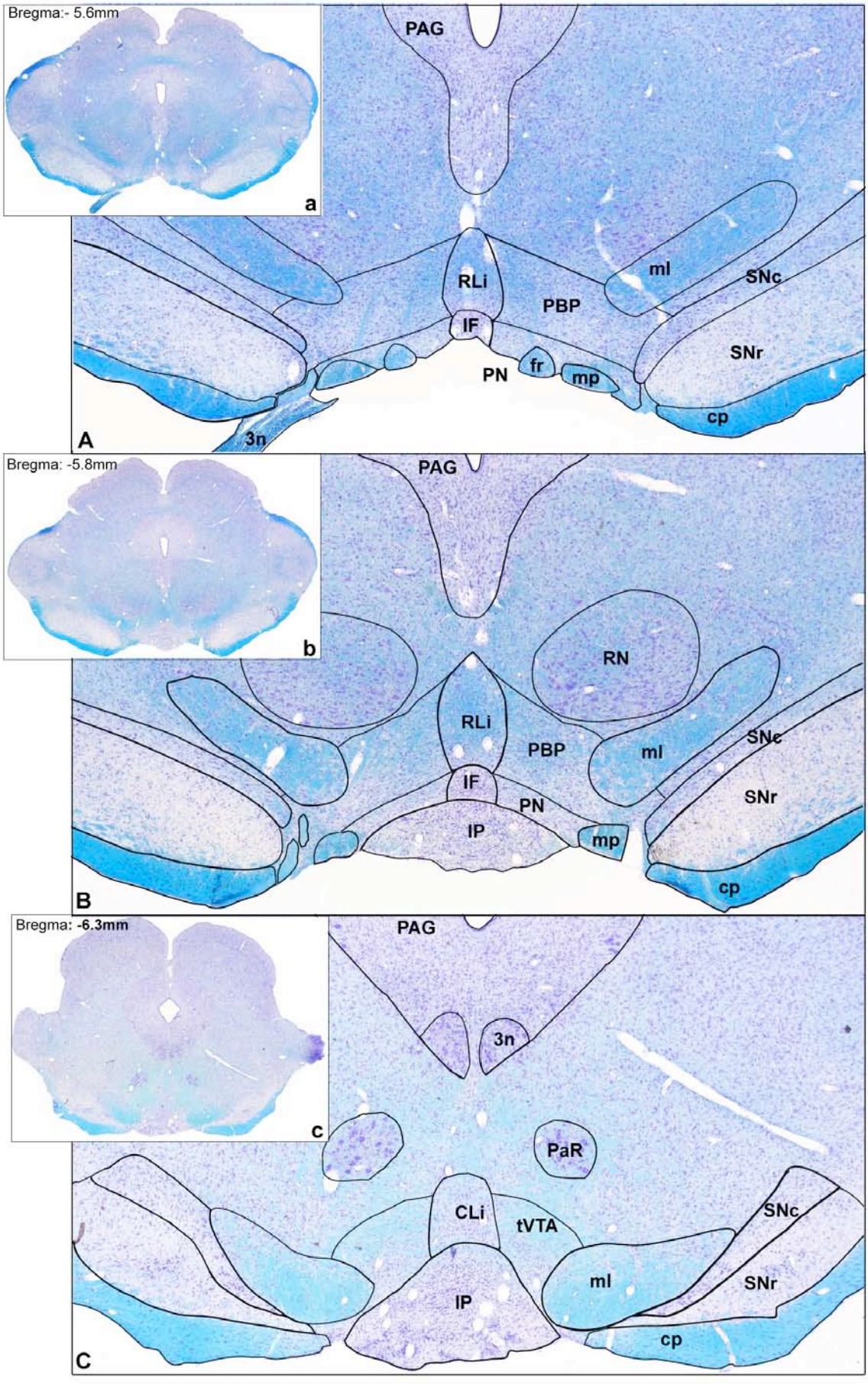

Bregma:- 5.6mm
a
PAG
RLi
IF
PBP
ml
SNc
SNr
fr
mp
PN
cp
3n
A
Bregma: -5.8mm
b
PAG
RN
RLi
PBP
ml
SNc
IF
PN
SNr
IP
mp
cp
B
Bregma: -6.3mm
c
PAG
3n
PaR
CLi
tVTA
SNc
ml
SNr
IP
cp
C

Figure 1: Klüver-Barrera stained sections (A-C) show the anterior and posterior part of the VTA with its neighbouring structures. The anterior VTA is characterized at bregma -5,6mm till -5,7mm, the posterior part at bregma -5.8mm to -6.3mm (Insets a-c). The VTA is located in the ventromedial region of the midbrain and ventral to the red nucleus (RN). This area is divided into six subnuclei: the paranigral nucleus (PN), the parabrachiopigmented nucleus (PBP), the intrafascicular nucleus (IF), the central linear nucleus (LC) (not shown), the rostral linear nucleus (LR) (not shown), and the mesopontine rostromedial tegmental nucleus (RMTg) known as the tail of the VTA (tVTA). The anterior part is surrounded by the substantia nigra (SN) with pars reticularis (SNr) and pars compacta (SNc). The fasciculus retroflexus (fr) is the afferent fibre bundle projecting to the VTA. The interpeducular nucleus (IP) borders the posterior part of the VTA and the tVTA. The IP receives afferents from the medial habenula complex (MHc) but not the LHb.

SNc – substantia nigra pars compacta, SNr – substantia nigra reticular part, ml – medial lemniscus, cp – cerebral peduncle, fr – fasciculus retroflexus, mp – mammillary peduncle, RLi – rostral lineal nucleus of raphe, 3n – third nerve, PaR – pararubral nucleus, PAG – periaqueductal gray, RN – red nucleus

1.3. The habenular complex is an important relay station connecting limbic forebrain and midbrain areas

The habenula is a small, paired structure located in the dorsal diencephalon and protruding into the third ventricle. It is divided into a medial (MHb) and a lateral (LHb) part and consists of a heterogeneous group of neurons containing a variety of neurotransmitters and neuromodulators (Geisler et al. 2003; Andres et al. 1999). Projections from the lateral habenular complex to the ventral mesencephalon are well known (Phillipson 1979; Herkenham and Nauta 1979; Oades and Halliday 1987; Geisler et al. 2007; Geisler and Trimble 2008; Jhou et al. 2009; Kaufling et al. 2009). Immunocytochemical, hodological and functional studies indicate that the structure of the LHb is complex (Fig. 2). Ten distinct morphological areas were established within the lateral habenular complex, five within its medial division and five within its lateral division (Andres et al. 1999). Based on morphology and chemoarchitectural criteria these areas represent different subnuclei (Geisler and Zahm 2005; Andres et al. 1999).

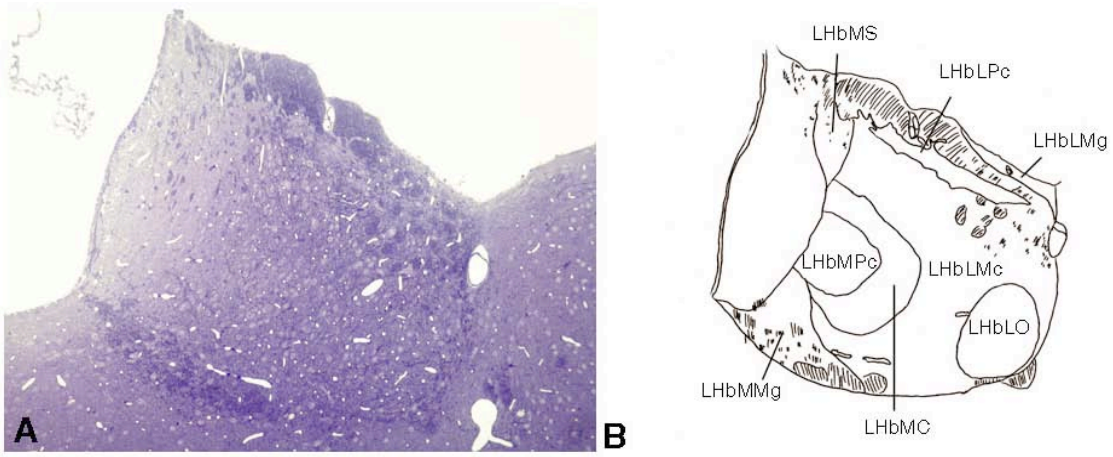


Figure 2: Based on the morphological analysis of semithin sections (A), recently, the LHb has been divided into ten distinct subnuclei, from which eight are shown in (B): LHbMS - superior subnucleus of the medial division of the LHb; LHbMPc - parvocellular subnucleus of the medial division of the LHb; LHbLPc - parvocellular subnucleus of the lateral division of the LHb; LHbLMg – marginal subnucleus of the lateral division of the LHb; LHbMMg - marginal subnucleus of the medial division of the LHb; LHbMC - central subnucleus of the medial division of the LHb; LHbLO - oval subnucleus of the LHb; LHbLMc - magnocellular subnucleus of the lateral division of the LHb; LHbLB - basal subnucleus of the LHb (not shown); LHbMA – anterior subnucleus of the medial division of the LHb (not shown).

Functionally, the LHb is described as a key relay station, which transmits information from limbic forebrain structures to midbrain nuclei and provides descending control over midbrain activity states. The LHb is involved in the regulation of a wide variety of biological processes like learning, sleep (Haun et al. 1992), sensitization to drug treatments (Funk and Stewart 1992), olfaction (Sutherland 1982), nutrition and nociception (Cohen and Melzack 1993; Mahieux and Benabid 1987; Fuchs and Cox 1993). The LHb is also critically involved in the regulation of female sexual receptivity, the hormonal induction of maternal behavior, and postpartum estrus behavior (Felton et al. 1999; Wagner et al. 1998; Metthews-Felton et al. 1995). Dysfunction of LHb signal transduction appears to be involved in the pathogenesis of schizophrenia (Sandyk 1992; Ellison 1994; Kelly 1998), depression (Caldecott-Hazard 1988; Winter et al. 2010) and drug addiction (Ellison 2002).

The biological importance of the LHb is emphasized by the fact that it is the only area showing strong activation in functional resonance imaging (fMRI) when human individuals become aware of performance errors (Ullsperger and von Cramon 2003; Shepard et al. 2006), suggesting that the LHb may reduce reward related feelings by suppressing the dopaminergic activity of the VTA. This idea received direct support by the fact that non-reward predicting stimuli resulted in activation of the habenular complex but caused an inhibition of dopaminergic neurons in monkeys. For reward predicting cues, this pattern was reversed (Matsumoto and Hikosaka 2007).

The LHb receives input from various limbic forebrain structures via the stria medullaris. Massive projections arise from the entopeduncular nucleus (EP) (Nagy et al. 1978), the rodent homologue of the primate internal segment of the globus pallidus (GPi). This pallido-habenular projection has been demonstrated in rats (Herkenham and Nauta 1977), cats (Nauta 1974) and monkeys (Parent 1979). Due to the principal involvement of the EP/GPi in the basal ganglia motor circuits, the pallidal afferences are considered to be the ”motor input” to the LHb. The pallido-habenular projections originate from the ventral pallidum (Sidibe et al. 1997). Furthermore, dense projections arise from the anterior lateral hypothalamic area, the lateral preoptic area, the bed nucleus of stria terminalis, the prefrontal cortex and from the central amygdaloid nucleus (Kowski 2008; Herkenham and Nauta 1977; Beckstead et al. 1979; Rajakumar et al. 1993). Afferences also arise from the septal regions, the nucleus of the diagonal band of Broca, the nucleus accumbens and from several brainstem nuclei. These comprise the serotoninergic neuronal groups of the median and dorsal raphe nuclei, the dopaminergic projections of ventral VTA, and the cholinergic cells of the laterodorsal tegmental nucleus of the ventral central gray (Herkenham and Nauta 1979; Phillipson and Pycock 1982; Skagerberg et al. 1984; Kalen et al. 1985; Behzadi et al. 1990).

1.4. The aim of the present study

Neurons in the LHb project to multiple structures in the CNS. These efferences form the mantle of the fasciculus retroflexus (fr) which is the major output path of the LHb. Descending efferents especially of the medial division of the LHb target the median and dorsal raphe nuclei, the periductal gray, and the locus coeruleus (Herkenham and Nauta 1977; Pasquier et al. 1977; Herkenham and Nauta 1979; Araki et al. 1984; Kalen et al. 1985; Oades and Halliday 1987). Anterogade tracing studies have also demonstrated projections to the substantia nigra (SN) and the VTA. The characteristics of LHb projections to the VTA have not been elucidated so far. Particularly the neurotransmitters involved in these projections and the nature of the targeted cells remain to be identified.

In the present study, we answer the following questions:

1. What kind of transmitter does the LHb use to influence the VTA?
2. What are the target structures in the VTA?

To answer these questions we combined tracing techniques with a wide array of histological methods to determine the cellular targets of the LHb afferences in the VTA and the neurotransmitters involved in the synaptic signal transmission process. From our results it is expected that they extend our understanding of the role of the LHb in the regulation of the midbrain dopaminergic system.

2. Material and Methods

2.1. Chemicals

Acetic acid	Merck	Darmstadt, Germany
Agarose	Serva	Heidelberg, Germany
Ammonium nickel sulfate	Sigma-Aldrich	Munich, Germany
Araldit CY212	Serva	Heidelberg, Germany
Bovine serum albumin – BSA	Sigma-Aldrich	Munich, Germany
3,3`diaminobenzidine tetrahydrochloride - DAB	Sigma-Aldrich	Munich, Germany
2-Dodecenylsuccinic acid anhydride - DDSA	Serva	Heidelberg, Germany
2,4,6-Tris-(dimethylaminomethyl)-phenol - DMP-30	Serva	Heidelberg, Germany
Disodiumtetraborate	Merck	Darmstadt, Germany
Entellan	Merck	Darmstadt, Germany
Ethanol	Merck	Darmstadt, Germany
Gelatin	Merck	Darmstadt, Germany
Glutaraldehyde	Sigma-Aldrich	Munich, Germany
Gold chloride	Sigma-Aldrich	Munich, Germany
Gum arabic	Merck	Darmstadt, Germany
Heparin	Ratiopharm	Ulm, Germany
Imidazol	Sigma-Aldrich	Mnich, German
Ketamin-50-curamed - Ketamine hydrocholoride	CuraMed	Karlsruhe, Germany

Longasteril 70	Fresenius	Bad Homburg, Germany
Normal goat serum – NGS	Interchem	Bad Kreuznach, Germany
Osmium	Roth	Karlsruhe, Germany
Paraformaldehyde	Sigma-Aldrich	Munich, Germany
Phosphate buffered saline – PBS	Merck	Darmstadt, Germany
PBS-Albumin – P-BSA	Sigma-Aldrich	Munich, Germany
Periodic acid	Merck	Darmstadt, Germany
Phenyl hydrazine	Merck	Darmstadt, Germany
2,4,6-trinitrophenol - Picric acid	Merck	Darmstadt, Germany
Propylene oxide	Serva	Heidelberg, Germany
Saccharose	Merck	Darmstadt, Germany
Silver Enhancement Kit	Amersham	Little Chalfont, UK
Sodium acid	Sigma-Aldrich	Munich, Germany
Sodium borohydride	Sigma-Aldrich	Munich, Germany
Sodium dihydrogen phosphate	Sigma-Aldrich	Munich, Germany
Sodium hydrogen phosphate	Merck	Darmstadt, Germany
Sodium metaperiodate	Merck	Darmstadt, Germany
Sodium thioglycolate	Merck	Darmstadt, Germany
Thimerosal	Sigma-Aldrich	Munich, Germany

Toluidine blue	Merck	Darmstadt, Germany
Triton X 100	Serva	Heidelberg, Germany
Tris(hydroxymethyl)aminomethane – Tris	Sigma-Aldrich	Munich, Germany
Uranyl acetate	Merck	Darmstadt, Germany
Xylene	J.T. Baker	Deventer, Holland

Sources of primary antibodies are depicted in Table 1. Biotin-labelled secondary antibodies and the ABC-Elite complex were from Vector (Vector Laboratories, Burlingame, CA, USA) and gold-labelled secondary antibodies from Nanoprobes (Yaphank, NY, USA).

Table 1. Dilutions of primary antibodies for immunocytochemistry at the light (LM) and electron microscopic (EM) level

Antibodies against	LM	EM	Host	Source	Address
GAD65	1:10000	1:2000	Rabbit	Chemicon	(1)
GAD67	1:10000	1:2000	Rabbit	Chemicon	(1)
vGluT1	1:10000	1:2000	Rabbit	UCSF neurology	(2)
vGluT1	1:100000	-	Guinea pig	Chemicon	(1)
vGluT2	1:5000	1:1000	Rabbit	UCSF neurology	(2)
vGluT2	1:100000	-	Guinea pig	Chemicon	(1)
PHA-L	1:10000	1:1000	Goat	Vector	(3)
Tetramethylrhodamine	1:500	-	Chicken	Author's laboratory	
GABA	1:5000	1:1000	Rabbit	Sigma	(4)

Tyrosine hydroxylase	1:10000	1:1000	Rabbit	Calbiochem	(5)
Digoxin, aP-labeled	1:5000	-	Sheep	Roche diagnostics	(6)

(1) Chemicon; supplier: Millipore GmbH, Am Kronberger Hang 5, 65824 Schwalbach/Taunus, Germany.

(2) University of California San Francisco, Department of Neurology, 505 Parnassus Ave, Box 0114, San Francisco, CA, 94143-0114, USA.

(3) Vector Laboratories, Burlingame, 30 Ingold Road, CA, 9401-2206, USA.

(4) Sigma-Aldrich Biochemie GmbH, Georg-Heyken-Strasse 14, D-21147 Hamburg, Germany.

(5) Merck Chemicals LTD; Padge Road, Beeston, Nottingham NG9 2JR, UK.

(6) Roche Diagnostics GmbH, Sandhofer Straße 116, D-68305 Mannheim, Germany.

2.2. Animals

All animal experiments were performed in accordance with the guidelines of the European Communities Council directive 86/609/EEC and were approved by the Regional Berlin Animals Ethics Committee (LaGeSo No. G 0168/01). Adult male and female Wistar rats (n=14; 230 to 320 g) were obtained from Charles River, Sulzfeld, Germany. Animals were housed in group-cages under controlled temperature (22°C) and illumination (12 hour cycle) with water and food ad libitum. After surgery animals were kept warm until fully recovered from anesthesia and then kept individually under the above mentioned conditions.

2.3. Tracer injections

Rats were deeply anaesthetized by intraperitoneal injections of a cocktail consisting of 45% ketamine (100 mg⁄ml; CuraMed, Karlsruhe, Germany), 35% xylazine (20 mg⁄ml; Bayer Vital, Leverkusen, Germany) and 20% saline, at a dose of 0.16 ml／100 g of body weight and placed into a Kopf stereotaxic instrument. For retrograde tracing experiments amounts of 1 µl of the retrograde tracer WGA-apoHRP-gold (E-Y Laboratories, San Mateo, CA, USA) were pressure-injected into mediocaudal areas of the ventral tegmental area. In anterograde tracing experiments Phaseolus vulgaris-leucoagglutinin (PhaL, 2.5% in 0.01M phosphate buffer, pH 7,4; Vector Laboratories, USA) or

tetramethylrhodamine conjugated dextran amine (TMR-DA, 2.5% in distilled water, pH 7.4; Invitrogen, Karlsruhe, Germany) was injected into medial aspects of the lateral habenular complex. These tracers were iontophoretically injected over 10-15 minutes through 1.0 mm glass micropipettes pulled to tip diameters of 10 µm (FG, CtB) or 15 µm (PhaL, TMR-DA). To avoid local tissuenecrosis a positive 1 µA (FG, CtB) or 4 µA (PhaL, TMR-DA) pulsed current (seven seconds pulse and interpulse duration) was used. Micropipettes were left for another 15 minutes in situ to avoid spread of tracer along the injection tract. Stereotaxic coordinates were adapted according to a broadly used atlas (Paxinos and Watson 1998). After surgery animals were kept warm in single cages until full recovery from anesthesia.

2.4. Fixation of animals by vascular perfusion

After 3 or 14 days of survival, rats were again deeply anaesthetized as described above using 200 IU heparin intraperitoneal. The perfusion began with a 10 second flush of a plasma substitute (Longasteril 70, Fresenius, Bad Homburg, Germany) following by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4 (PB) for 30 minutes.

Brains were dissected out, surrounded with agarose and cut into 1 - 5.5mm coronal blocks. The blocks were removed, cryoprotected in 0.4 M sucrose for about 4 hours and in 0.8 M sucrose overnight, shock-frozen in hexane at -70°C and stored at -80°C until use. Blocks of interest were cut into coronal 25 µm thick cryostat sections. The sections were either processed immediately or stored at -20°C in a cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1 M PB, pH 7.4) until use. Subsequent data are based on the analysis of about 500 individual sections derived from 12 rats.

2.5. Immunoperoxidase cytochemistry at the light microscopic level

Freely floating sections were rinsed in PBS, treated for 15 minutes with 1% sodium borohydride in PBS to destroy fixative-derived aldehyde groups, and thoroughly washed several times in PBS. Thereafter, sections were pretreated for 30 minutes in a blocking and permeabilizing solution, consisting of 10% normal goat serum (NGS; Interchem,

Bad Kreuznach, Germany), 0.3% Triton X-100 (Serva, Heidelberg, Germany), and 0.05% phenylhydrazine (Merck, Darmstadt, Germany) in PBS at room temperature (RT). Subsequent to the primary antibodies (dilutions are listed in table 1), applied for 36 hours in PBS containing 10% NGS, 0.3% Triton X-100, 0.1% sodium acid, and 0.01% thimerosal at 2°C. The sections were thoroughly rinsed in PBS, pretreated for 1 hour with 0.2% bovine serum albumin in PBS (PBS-A), and exposed for another 24 hours to the secondary antibody (biotinylated goat-anti-rabbit IgG, biotinylated goat anti-guinea pig IgG, or biotinylated goat anti-chicken IgG, Vector Laboratories, Burlingame, CA, USA), diluted 1:2,000 in 0.3% Triton X-100 and 0.1% sodium acid at RT. Subsequently, sections were repeatedly rinsed in PBS, and preincubated in PBS-A followed by 12 hours in an avidin-biotin elite complex solution (1:200 in PBS, Vector Laboratories, USA). After thorough rinses in PBS, preincubation for 15 minutes was performed in a solution of 0.05% diaminobenzidine (DAB) and 10 mM imidazole in 50 mM Tris buffer, pH 7.6. The visualization of the antigen-antibody-peroxidase complexes was started by the addition of 0.0015% hydrogen peroxide (25 µl of 0.03% hydrogen peroxide to 500 µl solution) and stopped after 15 minutes at RT by repeated washings with PBS. Sections were mounted onto gelatin-coated slides, air-dried not longer than 30 minutes, dehydrated through a graded series of ethanol, transferred into xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

2.6 Cresyl violet and methyl green stain

Slide-mounted sections were left in 70% ethanol overnight, rinsed in bidistilled water, and stained either with cresyl violet (0.2% cresyl violet acetate in 20 mM acetate buffer, pH 4.0) or methyl green (2% methyl green in 20 mM acetate buffer, pH 5.0) for 30 minutes at RT. After rinsing in bidistilled water, sections were dehydrated and coverslipped.

2.7. Immunoperoxidase cytochemistry at the electron microscopic level

Free floating coronal 25 µm thick cryostat sections or 50 µm thick vibratome sections were treated as mentioned above with the following exceptions. Only the blocking and permeabilizing solution contained Triton X-100 (0.1%), whereas the subsequent solutions were devoid of detergent.

2.7.1. Preparation and embedding of brain slices for electron microscopy

Immunoreacted sections were postfixed in 2% OsO4. After an increasing series of ethanol (70%, 80%, 90%, 96%, 100%, two changes of each), the sections were treated with propylene oxide (Serva, Heidelberg, Germany) for 5, 10 and 15 minutes. Then sections were placed into a mixture of araldite and propylene oxide (1:1, 24:30 w:w DDSA: CY212 Araldit, Serva, Heidelberg, Germany) overnight at RT. The next step is the incubation of the sections in araldite (24:30 w:w DDSA: CY212 Araldit) mixed with 2% DMP-30 for 2x2 hours. Overnight the sections were embedded in pure araldite. The tissue polymerizes at 65°C.

Semithin sections (500nm) and ultrathin sections (70nm) were cut using a diamond knife (Diatome, Biel, Switzerland) and a Leica Ultracut microtome (Reichert Ultracut S, Leica, Wetzlar, Germany). Semithin sections were dried and stained with 1% toluidine blue on a gelatine coated slide (1% toluidine blue, 40% saccharose, 1% di-sodium tetraborate dissolved in bidistilled water, pH 9.3) for 5 minutes at 70°C. Ultrathin sections were collected on 200 till 300 mesh nickel grids which were previously coated with 0.75% pioloform in chloroform. In order to contrast the ultrathin sections they were stained with uranyl acetate followed by lead citrate (Reynolds 1963). The ultrathin sections were examined with a Zeiss EM912 electron microscope.

2.7.2. Post-embedding immunocytochemistry on ultrathin sections

200-300 mesh grids with immuno-pretreated ultrathin sections were treated with 1% sodium periodate and 1% sodium metaperiodate as etching solution each for 5 minutes. Grids were rinsed in distilled water and TBS (mixture of 50mM Tris buffer and Triton X-100). The primary antibodies (rabbit anti GABA, Sigma) were then applied overnight at RT and at 1:5000 dilution in TBS containing 2% NGS, 0.03% Triton X-100. The next day the sections were washed with TBS several times. The secondary antibody (goat anti rabbit) was diluted 1:1000 in a solution with TBS and 2% normal goat serum for 4 hours at RT. Sections were then rinsed several times in TBS and finally rinsed in distilled water. In order to contrast the ultrathin sections they were stained with uranyl acetate followed by lead citrate and examined with a Zeiss EM912 electron microscope.

2.8. In-situ-hybridization

Riboprobe generation for VGLUT1-3 were described recently (Geisler et al. 2007). The cDNAs of GAD65 (GenBank Acc.No. M72422 extended by the EST CB785552, 535 bp) and GAD67 (GenBank Acc.No. M76177, 709 bp) were PCR amplified using Advantage Taq PCR mixture (Clontech, Hamburg, Germany) and the following primers: rat GAD65, 5'- GGAGTATGGGACCACAATGG-3' and 5'-TTTACTTTGTCCTTTTCCACTGC-3'; rat GAD67 5'-CAAGTTCTGGCTGATGTGGA-3' and 5'-GAGCGACATGCTGTGGTCTA-3'. The PCR products were cloned into the pGEM-T vector and sequenced. DIG-labelled antisense and sense probes were generated by linearizing the plasmid with *Not*I (GAD65 and GAD67) and either *Sac*II (GAD65) or *Sph*I (GAD67) and by using the DIG RNA labelling Kit (Roche Diagnostics, Mannheim, Germany). For in-situ-hybridization, freely floating sections were treated as described recently (Geisler and Zahm 2006) with minor modifications. In short, sections were rinsed in phosphate buffered saline (PBS: 0.15 M sodium chloride in 0.01 M phosphate buffer, pH 7.4), pretreated with sodium borohydride and acetic anhydride. After 30 minutes prehybridization, sections were temporarily mounted on clean slides, covered with 90 µl hybridization buffer containing the relevant RNA antisense or sense probes at appropriate concentrations (GAD65, 0.5 µg/ml; GAD67, 2,0 µg/ml; vGluT1, 1,0 µg/ml; vGluT2, 2,0 µg/ml; vGluT3, 0,5 µg/ml), coverslipped and hybridized over night at 56°C in a humid chamber. For stringency washes, freely floating sections were incubated with 0.1x standard sodium citrate buffer for 30 minutes at 56°C. After RNAse treatment (30 ng/ml) for 30 minutes at RT, the Dig-labelled RNA probes were visualized with anti-digoxigenin antibodies conjugated to alkaline phosphatase and the corresponding substrates nitroblue tetrazolium chloride and 5-bromo-4- chloro-3-indolyl phosphate. Sections were mounted on gelatin-coated slides, air-dried and coverslipped under Vectamount (Vector Laboratories Inc., Burlingame, CA, USA).

2.9. Silver intensification and gold-toning

Prior to immunocytochemical processing, sections were rinsed in PBS and subsequently treated for 30 minutes with 10% sodium thioglycolate to block tissue-derived sulfhydryl groups. After rinsing in 0.15 M sodium nitrate, sections were immersed twice for 20 minutes each in a mixture of solutions A and B of the IntenSE M Silver Enhance-

ment Kit (Amersham Biosciences, Little Chalfont, UK) for silver intensification of gold-labelled tracer molecules. Unspecifically tissue-bound silver ions were removed by fixation for 10 minutes in 5% sodium thiosulfate. After three additional washes in 0.15 M acetate buffer, pH 5.6, silver deposits were stabilized by gold toning with 0.05% gold chloride in acetate buffer for 7 minutes in an ice bath.

2.10. Immunofluorescence cytochemistry

Freely floating sections were rinsed in PBS, treated for 15 minutes with 1% sodium borohydride in PBS to destroy fixative-derived aldehyde groups, and thoroughly washed in PBS. Thereafter, sections were pretreated for 30 minutes in a blocking and permeabilizing solution consisting of 10% normal goat serum (NGS; Interchem, Bad Kreuznach, Germany) and 0.3% Triton X-100 (Serva, Heidelberg, Germany) in PBS at RT. Sections were treated with the primary antibodies (see table 1) for 36 hours at optimized dilutions in PBS containing 10% NGS, 0.3% Triton X-100, 0.1% sodium acid, and 0.01% thimerosal at 4°C. Subsequently they were thoroughly rinsed in PBS, pretreated for 1 hour with 0.2% bovine serum albumin in PBS (PBS-A), and exposed for another 24 hours to fluorochrome-labelled secondary antibodies (Alexa488-labeled goat anti-rabbit IgG and Alexa594-labeled goat anti-chicken IgG; Invitrogen, Karlsruhe, Germany), diluted 1:2,000 in 0.3% Triton X-100 and 0.1% sodium acid at RT. After repeated washings in PBS, sections were mounted onto gelatin-coated slides, shortly air-dried, coverslipped with MountFluor (Biocyc GmbH, Luckenwalde, Germany) and analysed with a Leica CS-SL confocal microscope.

3. Results

The inhibitory control of the lateral habenular complex (LHb) over the dopaminergic (DA) neurons in the VTA is well documented (see introduction). It is not known whether this inhibition is due to a direct inhibitory (GABAergic or glycinergic) influence or to an excitatory (cholinergic or glutamatergic) projection via inhibitory neurons in the ventral mesencephalon. Immunocytochemical screening of the LHb for glycinergic neurons with antibodies against the glycine transporter and for cholinergic neurons with antibodies against choline acetyl transferase or the vesicular acetylcholine transporter yielded no staining of cell bodies (not shown), ruling out glycinergic or cholinergic projections. Therefore the projection to the VTA most likely originates from GABAergic or from glutamatergic neurons in the LHb.

3.1. GABAergic neurons in the LHb are sparse

Primary attempts to identify GABAergic neurons in the LHb with antibodies against GABA itself (not shown) or against its synthesizing enzyme glutamate decarboxylase (GAD) were unsuccessful because the high density of the GABAergic axons and terminals in the LHb obscured the potential visualization of GAD-positive neurons (Fig. 3A, B). Instead, GABAergic neurons were successfully identified using in-situ-hybridization with anti-sense riboprobes to the mRNA of the 67kD isoform of the GAD enzyme (GAD67-mRNA) but not to the mRNA of the 65kD enzyme. The in-situ-hybridisation material was kindly provided by R. Lommel. Surprisingly, GABAergic neurons were sparse (Fig. 3C, D) and largely restricted to the oval subnucleus (Andres et al. 1999; Geisler et al. 2003) of the LHb. Additional data from our group (Dittgen et al. 2006) show that only minor projections to the VTA if any arise from this area, consequently, it appears very unlikely that the strong inhibition of the DA neurons in the VTA by afferents from the LHb (Ji and Shepard 2007) is due to a direct GABAergic projection.

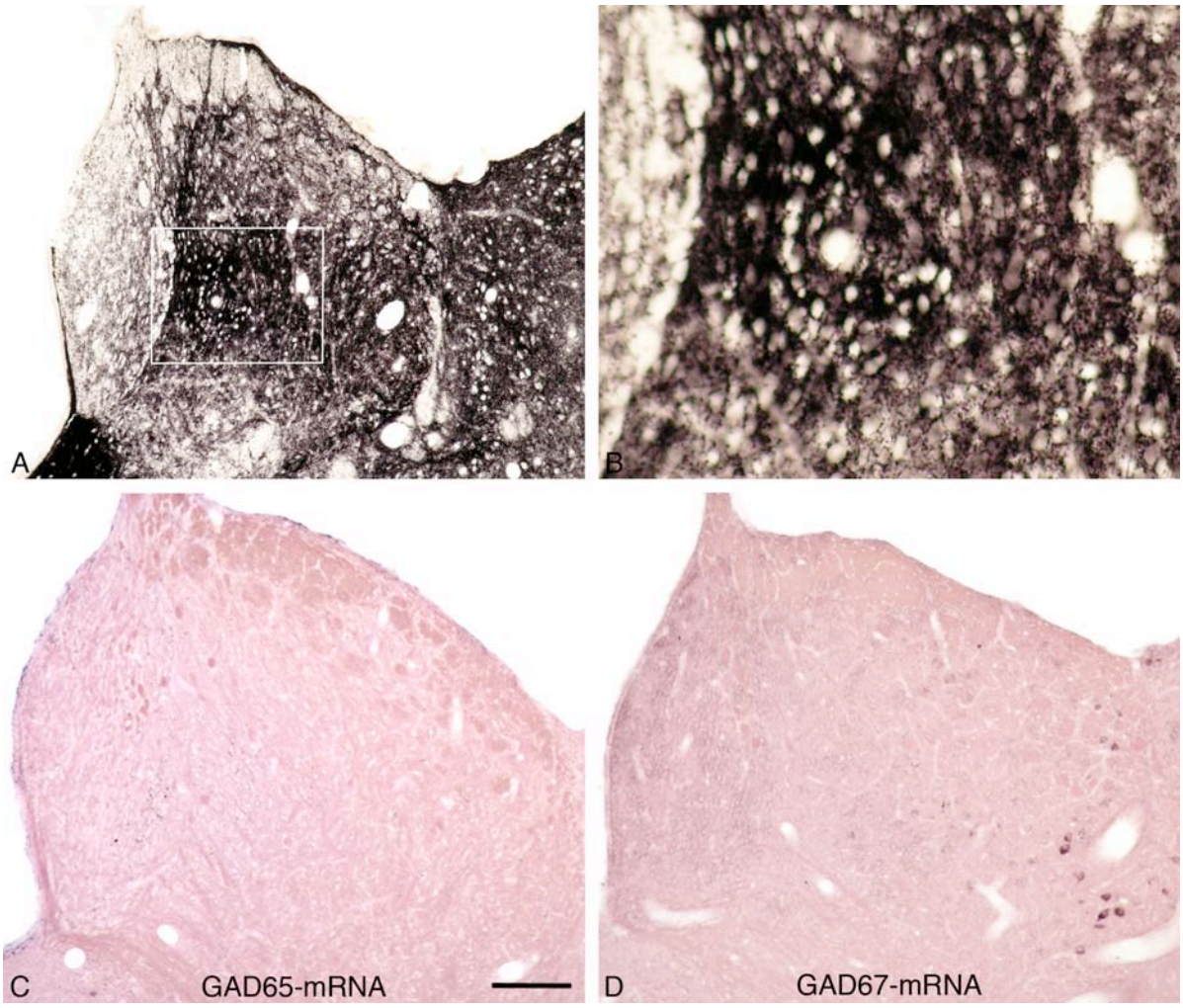


Figure 3: Glutamate decarboxylase (GAD) is a well known marker for GABAergic neurons and fibres. Visualization of GAD immunoreactivity (A, B) results in densely accumulated small structures, especially in the lateral habenula (LHb). At higher magnification (B) it becomes apparent that many neurons are negative. Thus, the heavy staining is due to fibres and terminals. Unfortunately, it precludes the unequivocal identification of any potentially GAD-positive neurons. GAD exists as two isoforms with molecular weights of 65 kD or 67 kD, which are products of two distinct genes. GABAergic neurons can be identified with the aid of digoxigenin-labelled anti-sense probes to the GAD65-mRNA or the GAD67-mRNA using in-situ-hybridization. While no GAD65-mRNA (C) could be detected, a few neurons in the lateral LHb contained GAD67-mRNA (D). They were concentrated in an area, which matches the localization of the oval subnucleus of the LHb. Scale bar in (C) indicates 190 µm for (A), 630 µm for (B), and 125 µm for (C) and (D).

3.2. Most neurons in the LHb are glutamatergic and use vGluT2 to fill their synaptic vesicles

In agreement with recent data (Varoqui et al. 2002), attempts to use antibodies against vesicular glutamate transporters (vGluTs) to identify glutamatergic neurons in the LHb also were unsuccessful. While the MHb displayed prominent vGluT1 immunoreactivity, staining in the LHb was generally weak and showed no cells at all (Fig. 4A, B). In contrast, vGluT2 immunoreactivity in the LHb was very intense occluding the visualization of potential glutamatergic neurons (Fig. 4C, D).

In-situ-hybridization experiments were again helpful. Most areas of the medial habenular complex were densely packed with neurons containing vGluT1 mRNA. These cells, however, could not be detected in the LHb (Fig. 4E), where most neurons expressed vGluT2 mRNA (Fig. 4F). These data suggest that the output from the LHb is predominantly glutamatergic.

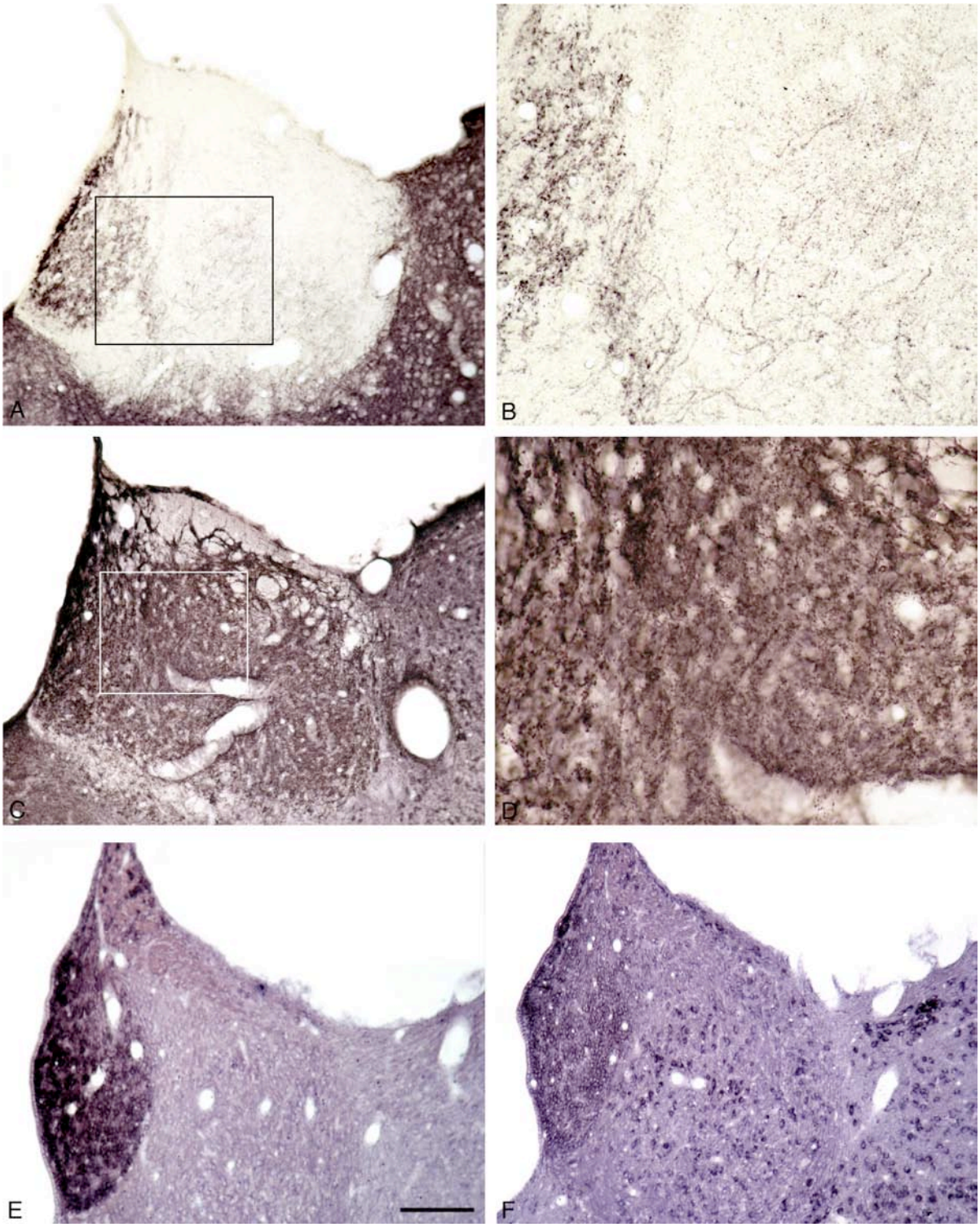


Figure 4: Immunocytochemical visualization of the vesicular glutamate transporter-1 (vGluT1; A, B) as a marker for glutamatergic neurons displayed clear staining in the medial habenular complex (MHb), while the LHb remained largely negative. In contrast, staining for vGluT2 (C, D) was prominent in the MHb as well as in the LHb. Unfortunately, the high density of vGluT2-positive fibres again precluded an unequivocal identification of potentially glutamatergic neuronal cell bodies in the LHb. In-situ-hybridization, however, demonstrated that the expression of vGluT1-mRNA was restricted to the MHb (E). In contrast, most neurons in the LHb contained vGluT2-mRNA in their cytoplasms (F). Thus, most neurons in the LHb ap-

pear to be glutamatergic. Scale bar in (E) indicates 200µm for (A), (C), (E), and (F), and 680 µm for (B) and (D).

3.3. Many neurons in the LHb project to the ventral mesencephalon

Next, we wanted to rule out a potential GABAergic connection from the LHb to the ventral mesencephalon, and the hypothesis of a glutamatergic projection needed additional support. For this purpose, individual LHb neurons sending fibres to the VTA or the rostromedial tegmental nucleus (RMTg) were visualized and their transmitter identified.

Projections from the LHb as well as from other areas to the VTA are well established (Phillipson 1979; Herkenham and Nauta 1979; Oades and Halliday 1987). How intense these are, however, was appreciated only quite recently, when ApoHRP-WGA-gold (Basbaum and Memetrey 1987) as a most sensitive retrograde tracer was applied (Geisler and Zahm 2005; Geisler et al. 2007). To determine which transmitter is used in the projection from the LHb to the ventral midbrain, ApoHRP-WGA-gold was combined with in-situ-hybridization. These slices were kindly provided by C. Gruber and A. Dittgen. Subsequent to pressure injection of the tracer into several areas of the VTA or the RMTg (Fig. 5A, inset), corresponding neurons in the LHb are identified by black dots in their cytoplasm representing silver-intensified gold granules (Fig. 5A, B, C). All retrogradely labelled cells simultaneously displayed the blue phosphatase reaction product for vGluT2 mRNA, unequivocally demonstrating that the LHb-VTA projection is glutamatergic (Fig. 5B, C). Other neurons with vGluT2 mRNA were devoid of retrograde label (Fig. 5B) and may project to different areas of the VTA.

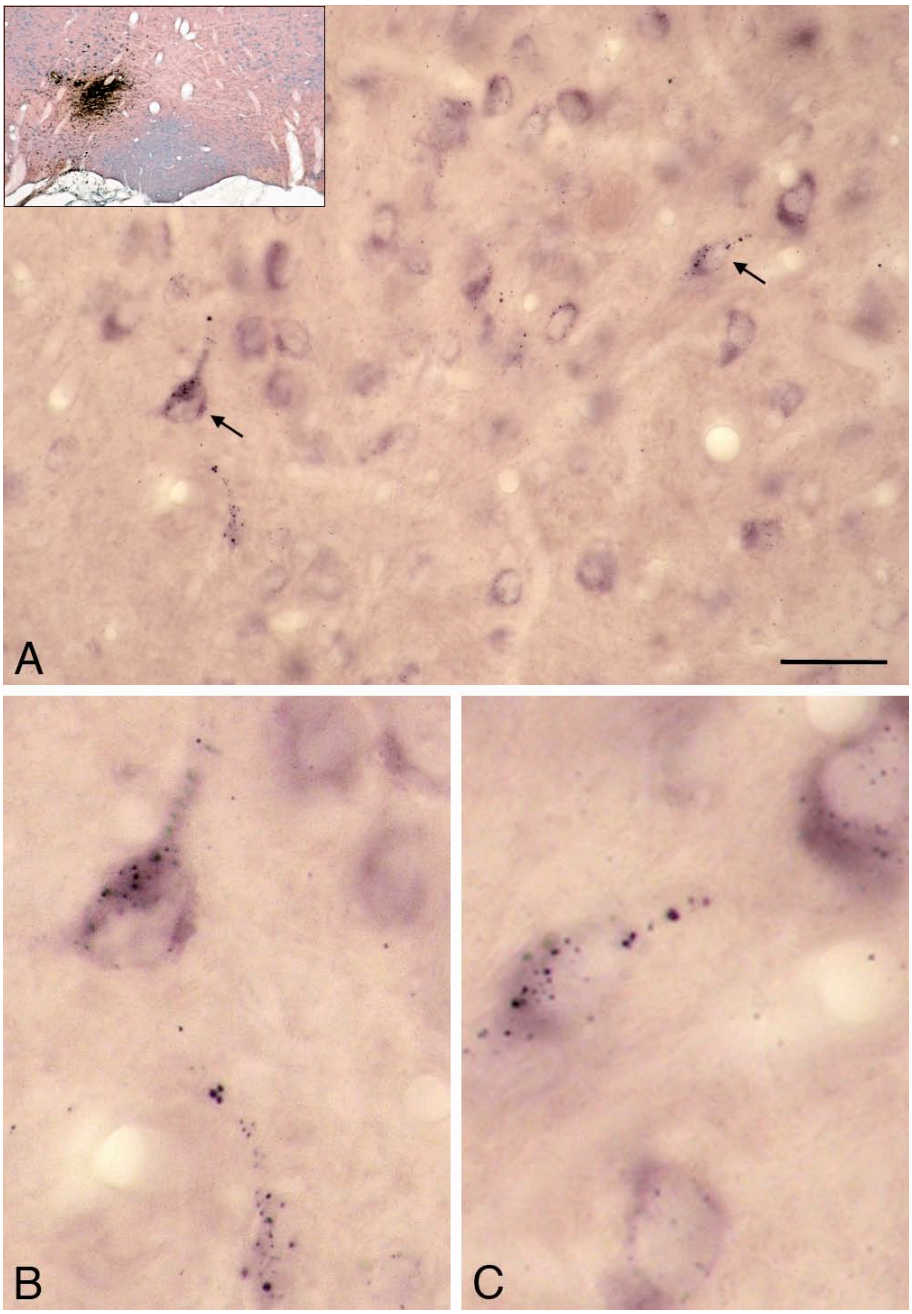
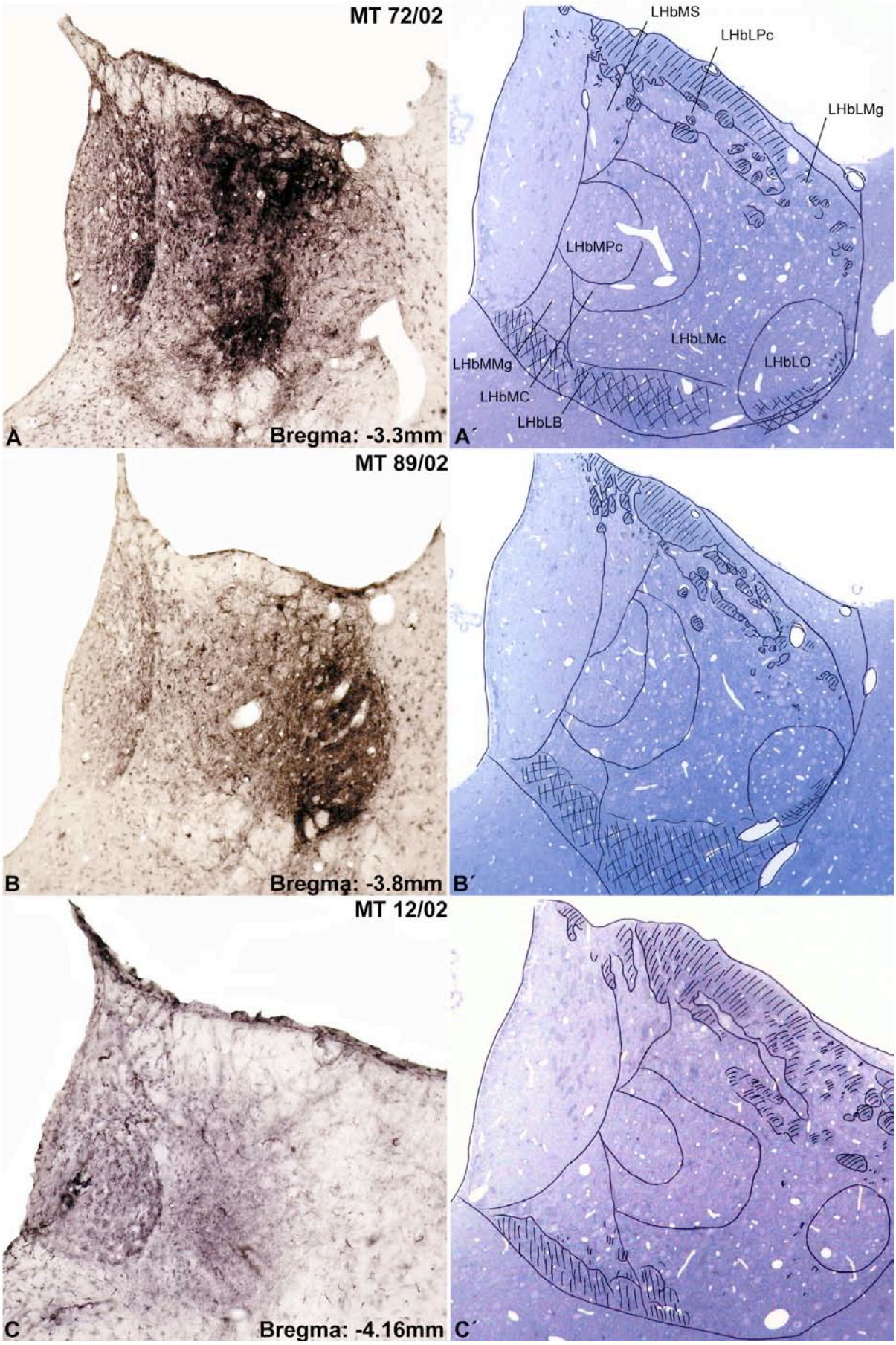


Figure 5: To learn which transmitter is used by those LHb neurons projecting to the VTA, a sensitive retrograde tracer, ApoHRP-WGA-gold, was injected into the VTA (A, inset). Two retrogradely labelled LHb neurons (A, arrows) are shown at higher magnification (B, C). The retrograde tracer is recognized by black dots in their cytoplasm (B, C) representing silver-intensified gold granules. Note, that all retrogradely labelled neurons simultaneously display blue reaction product indicating the presence of vGluT2 mRNA. Scale bar in (A) indicates 30 µm for (A), and 80 µm for (B) and (C).

3.4. Axons projecting from the LHb to the ventral midbrain use glutamate as synaptic transmitter

Unfortunately, the complex composition of the LHb had not been recognized at the time of these studies. Thus, it is currently not known, whether the fibres to the ventral mesencephalon randomly originate in the LHb, or whether they are predominantly derived from one or more subnuclei in the LHb.

From 17 rats with anterograde tetramethylrhodamine (TMR-DA) injections, five were selected for the present study and show different injection sites in the LHb (Fig. 6). Traced slices were kindly provided by A. Lehmann. In the case of MT 11/02 (Fig.6E), there is a small injection site on the lateral rostral part of the LHb in the large LHbLMc, the magnocellular subnucleus with bregma –3.8mm. Case MT 70/02 (Fig. 6D) represents a small and defined injection site in the area of the LHbMS, the superior subnucleus of the medial division of the LHb near the stria medullaris. Bregma –3.8mm. Case MT 72/02 (Fig. 6A) shows a large injection area along the LHb. The injection site lies more caudally in the habenula with bregma –3.3mm. Case MT 12/02 (Fig. 6C) shows its injection in the area of the LHbMS, the parvocellular subnucleus the LhbMPc, the central subnucleus of the medial division in the more caudal part of the LHb with bregma –3.3mm. Case MT 89/02 (Fig. 6B) shows a large injection site in the caudal, lateral division of the LHb in the area of the LHbLPc, the magnocellular subnucleus and the LHbLMC, the oval subnucleus with bregma –3.7mm.


MT 72/02
A
Bregma: -3.3mm
LHbMS
LHbLPc
LHbLMg
LHbMPc
LHbLMc
LHbMMg
LHbLO
LHbMC
LHbLB
A´
MT 89/02
B
Bregma: -3.8mm
B´
MT 12/02
C
Bregma: -4.16mm
C´

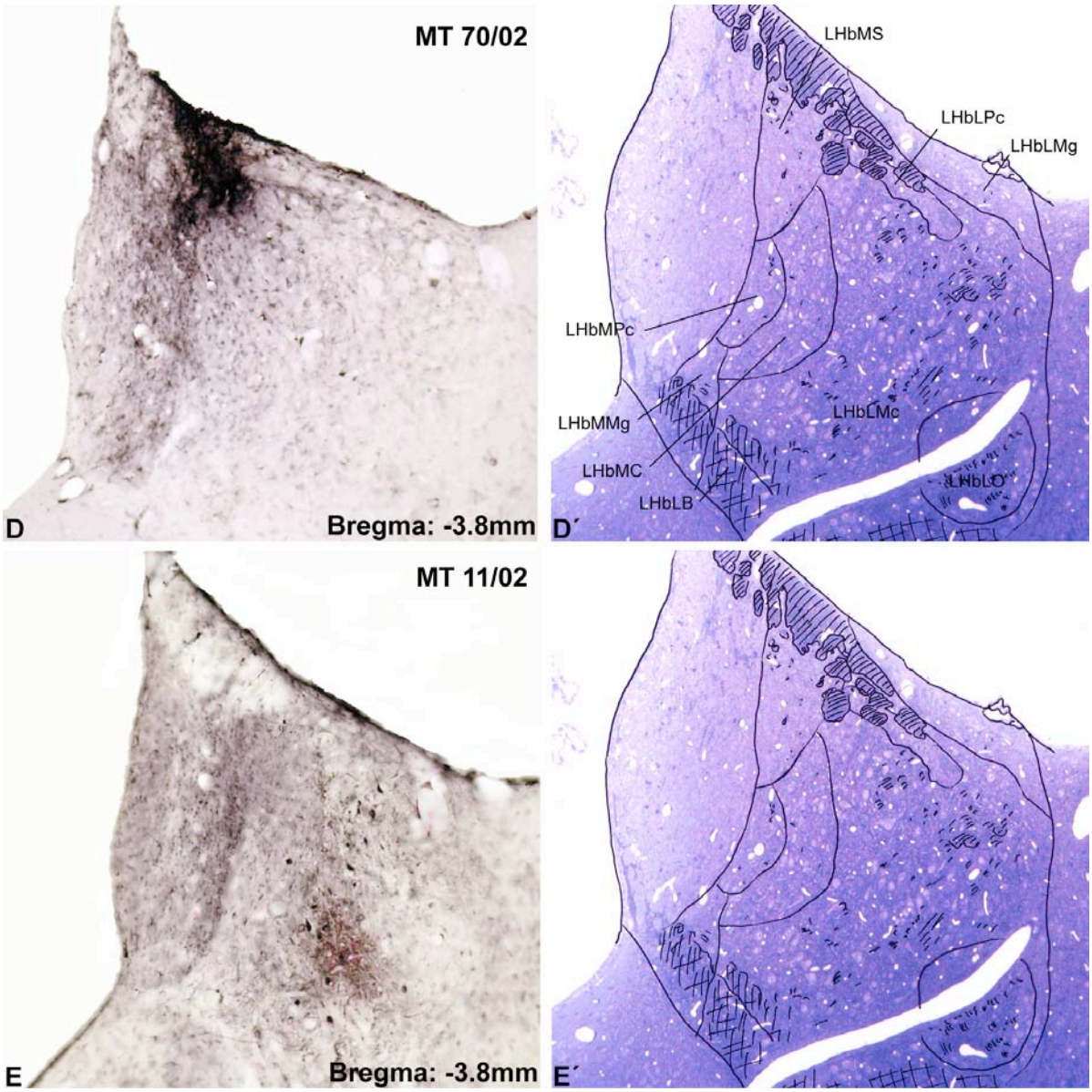


Figure 6: A-E show the habenula injection sites of five rats traced with the anterograde tracer TMR-DA. The injections are located in different subdivisions of the LHb. The cytoarchitecture of the LHb with its subnuclei organization is described in adjuvant semithin sections (A´-E´) created by Geisler 2003. Based on analysis of semithin sections, Geisler described ten distinct subnuclei within the lateral habenula. The lateral habenula is divided into subnuclei of the medial division and lateral division which are involved in distinct biological functions (Geisler 2003).

Animal MT-11/02 (Fig. 6E, Fig. 7) can be taken as a representative case to describe our results in greater detail. The injection is centered in the lower part of the magnocellular subnucleus of the LHb (Fig. 7A) as judged from a corresponding semithin section (Fig. 7B). Anterogradely labelled fibres with terminal-like specializations are distributed throughout the VTA extending from most rostral regions to areas overlapping with the RMTg (Fig. 7C, inset), and in the interpeduncular nucleus (see discussion).

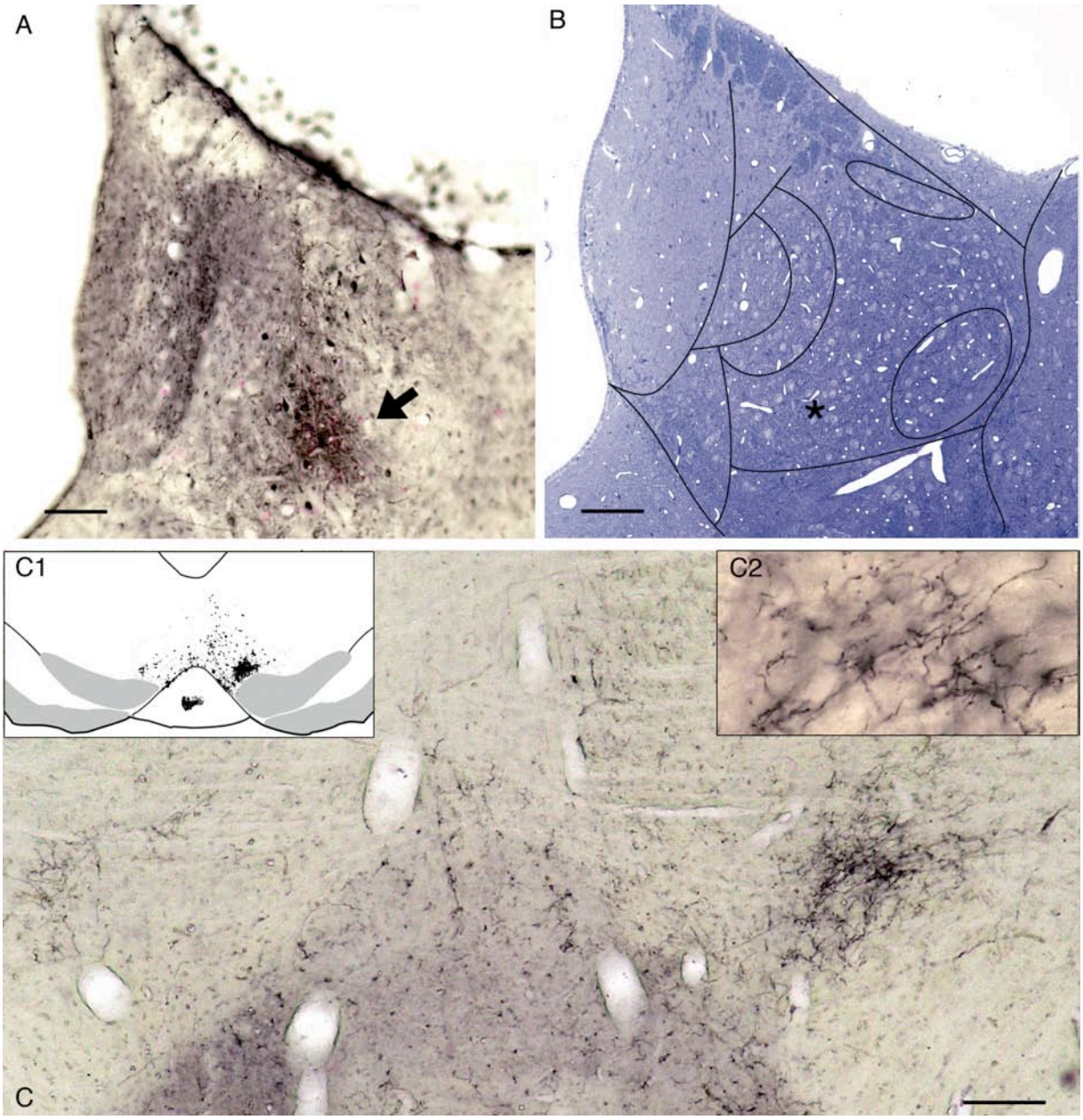


Figure 7: In animal MT-11/02, which is shown here as a representative example (see text), the anterograde tracer was injected into the ventral part of the magnocellular subnucleus of the LHb (A, arrow) as judged from a corresponding semithin section (B; asterisk presents injection side; lines indicate borders between subnuclei). Labelled terminals (C, inset 1) are distributed over the parabrachial pigmented, the paranigral, and the interfascicular subnuclei of the VTA, the tVTA, and the interpeduncular nucleus (C, inset 1). Terminal-like specializations are recognized at higher magnification (C, inset 2). Scale bars indicate 125 µm in (A) and (B), and 100 µm in (C).

3.5. The anterograde labelled habenular fibres displayed vGlut2 in terminal specialization in confocal analysis

When LHb terminals in the ventral midbrain were analysed for their potential transmitter, confocal analysis of sections doubly labelled for the tracer and selected transmitter markers revealed that LHb-derived axons were devoid of GAD- and vGluT1-immunoreactivity (Fig. 8A, B). In contrast, a considerable number of anterogradely labelled fibres displayed vGluT2-positive puncta (Fig. 8C). Certainly such dots were not very frequent. When taking into account, however, that only few axons are labelled with the anterograde tracer and that only few synapses will show up in the focusing plane of the microscope, this is what can be expected.

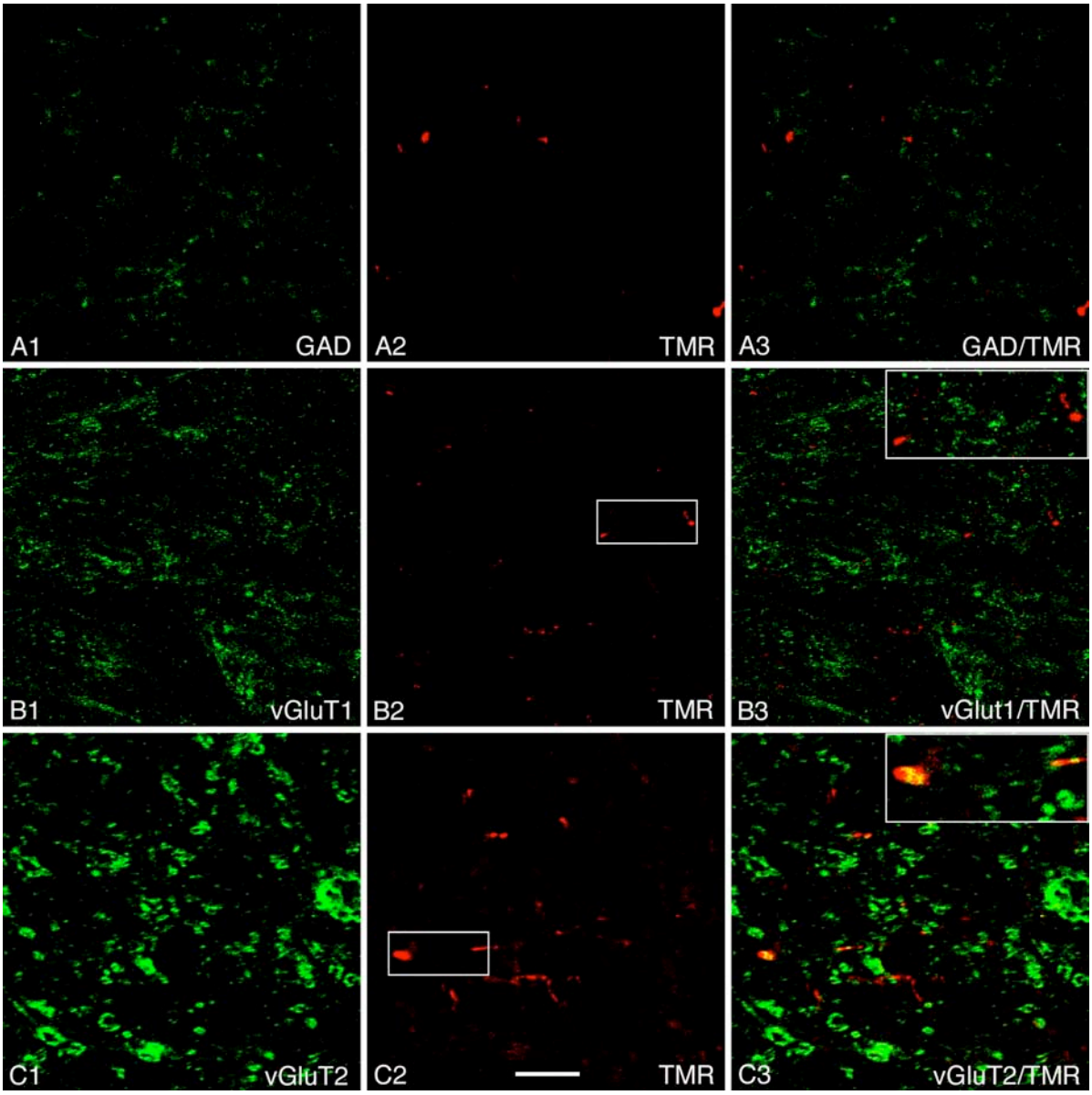


Figure 8: Axons containing TMR as anterograde tracer and potential transmitter markers are visualized by double immunofluorescence confocal microscopy. Green GAD- (A1), vGluT1- (B1), and vGluT2- (C1) immunoreactivities were more or less evenly distributed throughout the parabrachial pigmented subarea of the VTA, which contained several anterogradely traced axons in every section (A2, B2, C2). Neither GAD- nor vGluT1-staining could be detected over red-labelled axons (A3, B3). In contrast, merging of vGluT2-staining with TMR visualization (C3) resulted in yellow spots (synapses) in red-labelled axons, supporting the glutamatergic nature of the projection from the LHb to the VTA. Selected areas from (B2) and (C2) are shown after merging as insets in (B3) and (C3). Scale bar in (C2) indicates 20 µm for (A1 to A3) and (B1 to B3), and 10 µm for (C1 to C3).

3.6. Double labelling immunoelectron microscopy confirms that LHb fibres and terminals in the ventral mesencephalon use glutamate and not GABA as transmitter

In the present set of experiments the fibres from the LHb and their terminals were visualized in the electron microscope via the dark, diffuse product of polymerized diaminobenzidine (DAB product, Fig. 9A-D). LHb traced fibres create different shape and size of terminal structures in the VTA.

In immunodouble labelling at electron microscope level, the irregular shaped black dots represent vGluT2 immunoreactivity (Fig. 10A, B). When visualized simultaneously, all LHb-derived fibres or terminals robustly display black vGluT2 dots, while additional terminals without tracer staining (Fig. 10B; arrows) represent further excitatory terminals of unknown origins.

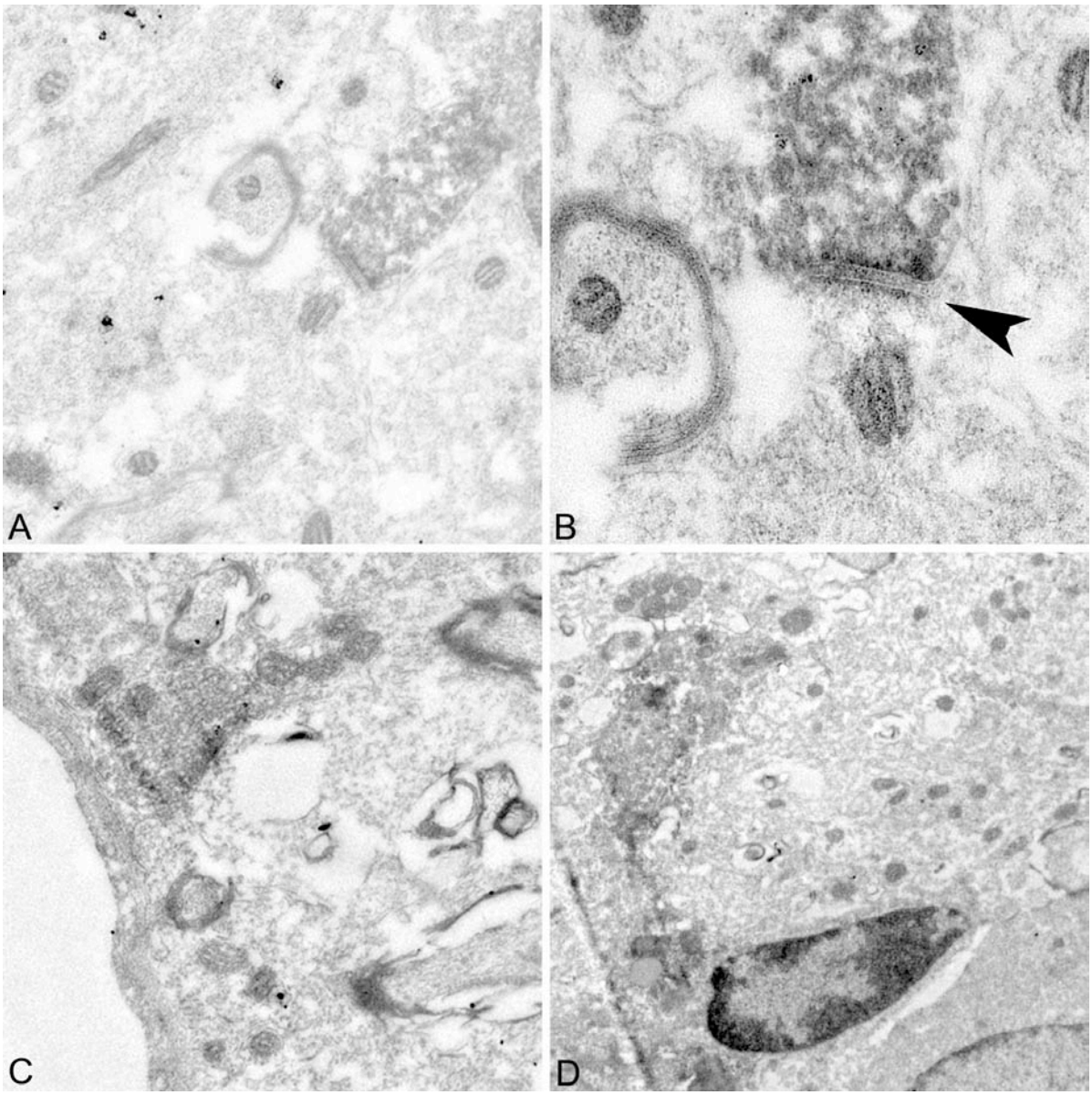


Figure 9: Terminals from the lateral habenula in the VTA are visualized with the DAB reaction product. The terminal structures show different shape and size (A, C, D). VTA terminals create asymmetric synapses (A, B) with large dendritic structures (arrowhead in B) as well as dendritic spines (not shown).

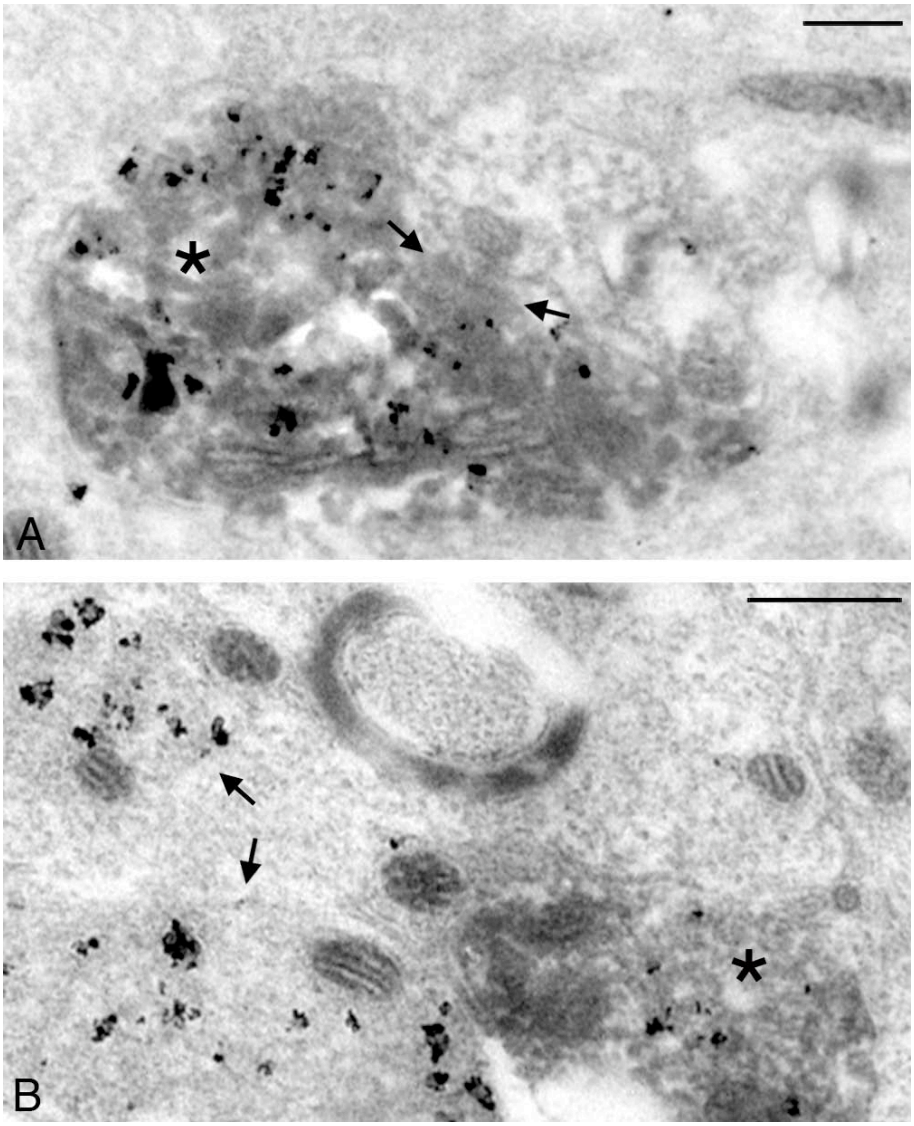


Figure 10: At the electron microscopic level, LHb fibres containing transported TMR-aminodextran were identified in the VTA by the immunoperoxidase technique via the diffusely dark DAB product in their axoplasm (A, B; asterisk). In double stained sections, irregular shaped black dots represent silver-intensified gold granules indicating vGluT2-immunoreactivity. Arrows indicate postsynaptic density in (A). All LHb-derived fibres contained vGluT2. However, some vGluT2-positive synapses are free of the DAB product (B, arrows) and may represent glutamatergic terminals derived from other areas of the brain. Scale bars indicate 250 nm in (A) and 500 nm in (B).

3.7. Most LHb fibres target on GAD- or GABA-positive profiles in the VTA or the RMTg

In contrast, GABAergic presynaptic structures as identified by their GAD65 immunoreactivities or GABA antibodies never contained tracer label (Fig. 11, A, B; 12 A-F). In line with the absence of GAD65 mRNA in the LHb, they either represent terminals of inhibitory neurons in the VTA or RMTg or may represent GABAergic inputs from other sources. Excitatory and inhibitory inputs may coincide on the same dendritic structure. Thus, GAD65-negative terminals from the LHb and GAD65- or GABA-positive terminals of unknown origin target the same dendrites (Fig. 11A, B, D) as seen in electron microscopic triple labelling experiments. Labelled synaptic terminals show different sizes of LHb synaptic specialization contacting dendritic structures (Fig. 12A-F).

Further double labelling experiments revealed that DAB-positive fibres from the LHb mostly terminate on large GAD67-positive dendrites (Fig. 11E, F). Prominent postsynaptic densities (Fig. 11B, E, F, arrowheads) characterize these synapses as asymmetric. This is in line with the notion that glutamatergic input from the LHb excites inhibitory neurons in the ventral midbrain.

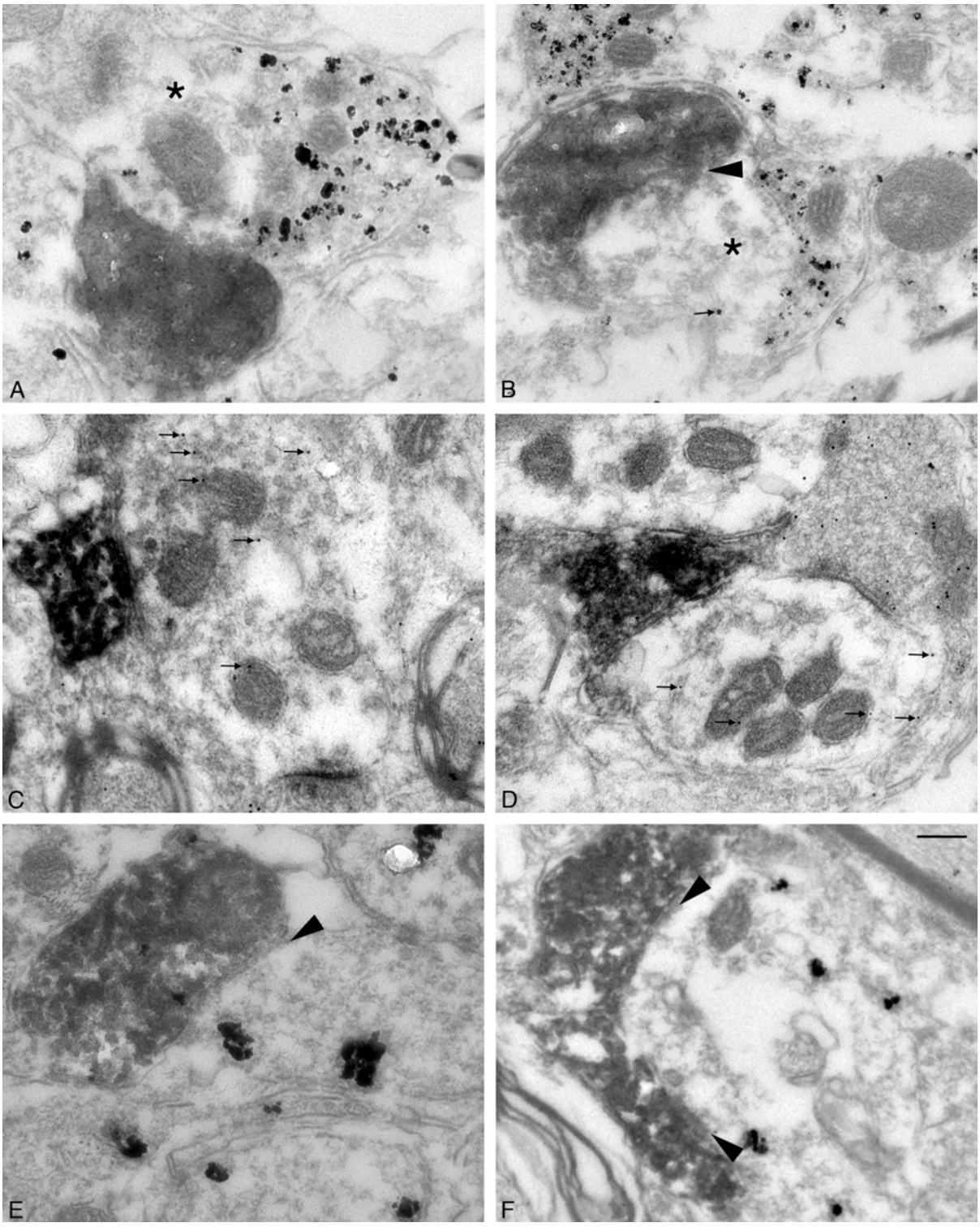


Figure 11: Labelled axon terminals from the LHb in the VTA never displayed GAD65- (A, B) or GAD67-immunoreactivity (E, F), as seen from electron microscopic double labelling for TMR-aminodextran (DAB) and GAD65 or GAD67 (silver-intensified gold granules). LHb terminals contacted dendrites, which very weakly displayed GAD65-immunoreactivity (A). Note that the same dendrite (A, B; asterisks) can be contacted by labelled axons from the LHb and GAD65-positive synapses of unknown origins. Unfortunately, even triple staining including post-embedding staining with anti-GABA antibodies did not yield convincing results, as postsynaptic GABA-staining (10 nm gold granules indi-

cated by small arrows in B to D) in this material is rather weak (compare staining intensities of pre- and postsynaptic compartments in D). However, most dendrites postsynaptic to labelled axons clearly contained GAD67- immunoreactivity (E, F) indicating the GABAergic nature of habenular targets in the VTA. Moreover, prominent postsynaptic densities (B, E, F; arrowheads) support the hypothesis that habenular terminals in the VTA are excitatory. Scale bar in (F) indicates 250 nm for all photographs.

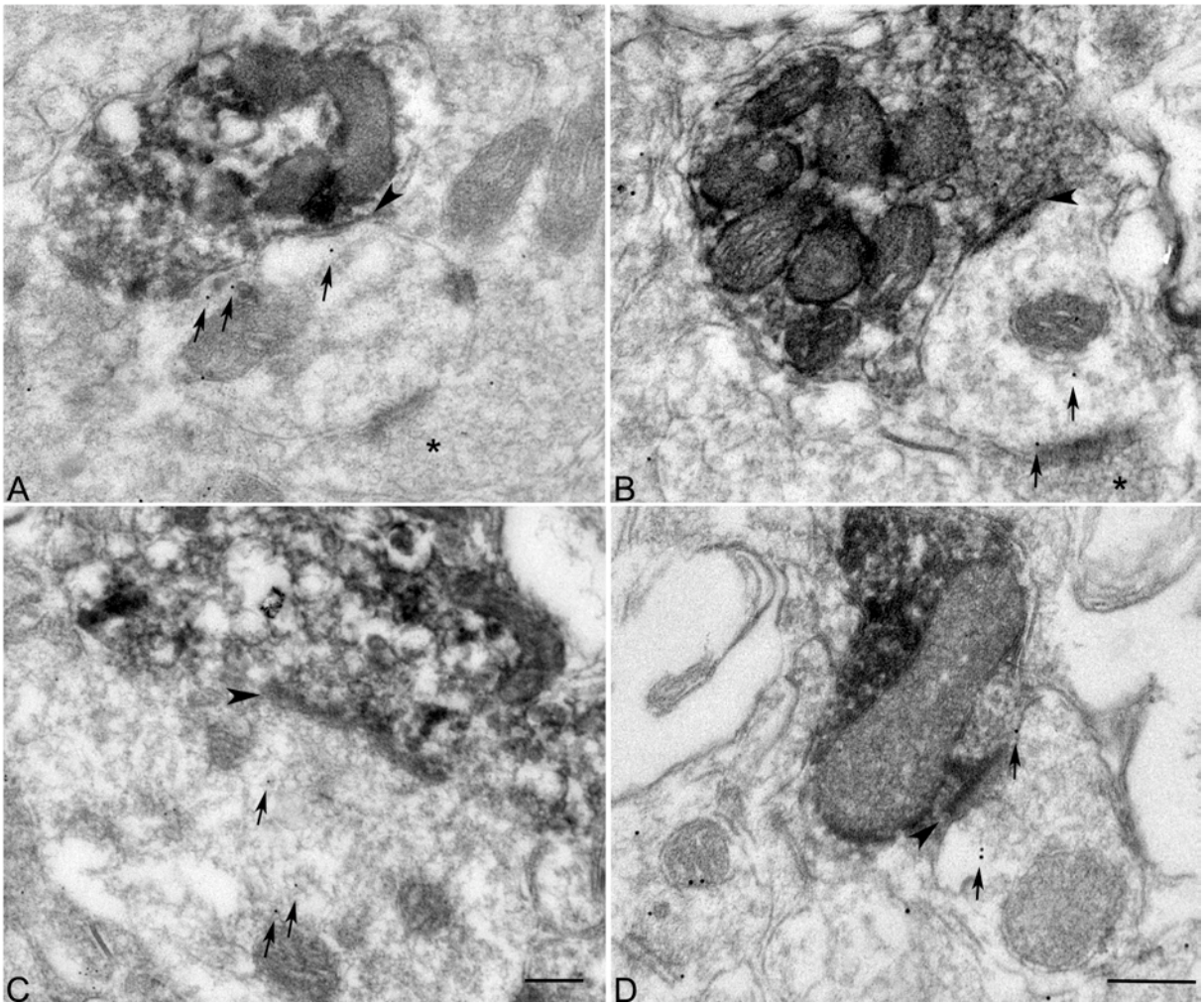


Figure 12: Other examples of LHb terminals synapsing on GABAergic dendrites. LHb terminals build synapses with GABA positive dendrites (gold granules marked with small arrows in A-D). The prominent postsynaptic densities support the idea of excitatory synapses (A-D; arrowheads). Scale bar in (C) indicates 250nm, scale bar in (D) indicates 250nm for the other photographs (A, B, D).

3.8. A small number of LHb axons terminate on dopaminergic neurons in the VTA

When looking for dopaminergic (TH-positive) structures, we found that most postsyaptic compartments targeted by incoming LHb axons in the VTA were devoid of labelling (Fig. 13A). However, there were a few terminals, which did contact TH-immunoreactive structures (Fig. 13B). Analysing 80 synapses with tracer-labelled terminals we counted 9 postsynaptic compartments containing TH-immunoreactivity. Thus, a small amount (about 10%) of incoming LHb fibres may directly activate DA neurons in the VTA.

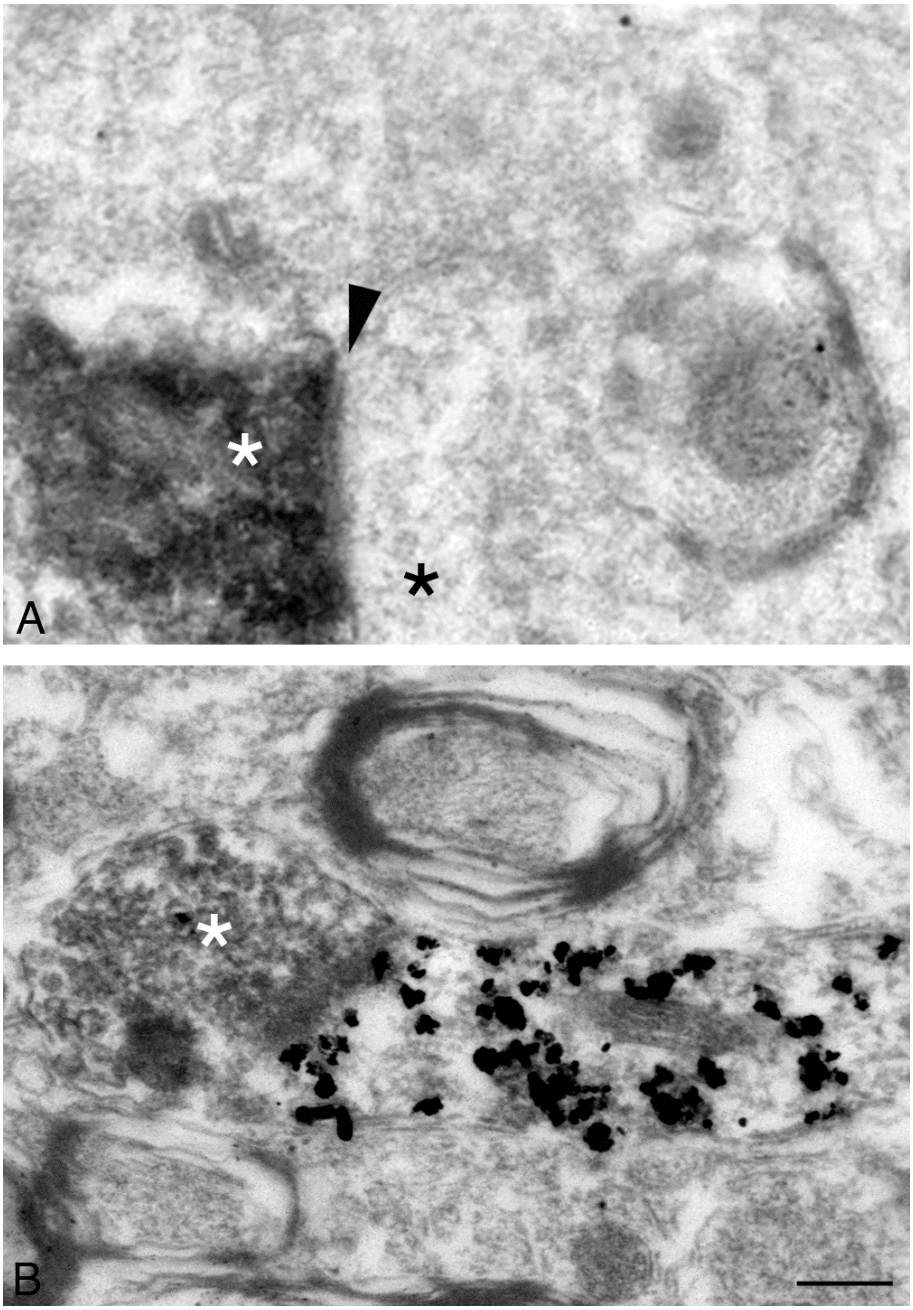


Figure 13: At the electron microscopic level, LHb fibres containing transported TMR-aminodextran were identified in the VTA by the immunoperoxidase technique via the diffusely dark DAB product in their axoplasm (A, B; asterisk). In double-stained sections, irregular-shaped black dots represent silver-intensified gold granules indicating vGluT2-immunoreactivity. Arrows indicate post-synaptic density in (A). All LHb-derived fibres contained vGluT2. However, some vGluT2-positive synapses are free of the DAB product (B, arrows) and may represent glutamatergic terminals derived from other areas of the brain. Scale bar indicates 250 nm in (A) and 500 nm in (B).

4. DISCUSSION

The prominent projections from the lateral habenular complex (LHb) to the ventral mesencephalon are well known (Phillipson 1979; Herkenham and Nauta 1979; Oades and Halliday 1987; Geisler et al. 2007; Geisler and Trimble 2008; Jhou et al. 2009a, b; Kaufling et al. 2009). In agreement with previous reports (Araki et al. 1988), we found that midbrain terminals from the LHb primarily project to the VTA and only slightly to the medial aspects of the SNc (see Fig. 7C). Early investigations suggested a tonic inhibitory influence of the LHb on cerebral dopamine transmission (Nishikawa et al. 1986). Results contradicting these findings were reported in studies, demonstrating that in vitro most VTA neurons responded with excitatory postsynaptic potentials to the stimulation of the LHb (Matsuda and Fujimura 1992). The present investigation provides ample evidence that the LHb-dependent inhibitory activity in the VTA is due to an excitatory glutamatergic projection of LHb neurons, targeting inhibitory GABAergic neurons in the VTA.

4.1. Methodological considerations

To identify transmitters used by LHb neurons projecting to the VTA, a combination of retrograde tracing and in-situ-hybridization is necessary. WGA-apoHRP-gold (Basbaum and Menetrey 1987) is a highly sensitive retrograde tracer, which can be clearly visualized in addition to non-radioactive in-situ-hybridization (Jongen-Relo and Amaral 2000) and is superior to other combinations (Geisler and Zahm 2006). WGA-apoHRP-gold needs to be applied by pressure injection. Consequently, it cannot be excluded that a few axons running through the VTA were damaged, resulting in false-positive retrogradely labelled neurons. Recent data, however, suggest that this effect may be less important (Geisler and Zahm 2006) for the present investigation.

In our anterograde tracing experiments, the fibres of passage problem is evident. Even when the tracer injection was restricted to the central area of the LHb (Fig. 7A) there were some labelled terminals in the interpeduncular nucleus (IP), which is known to receive fibres from the MHb and not from the LHb (Herkenham and Nauta 1979; Araki et al. 1988). Many axons from the MHb, however, pass through the LHb before joining the fasciculus retroflexus. Severing these axons during tracer injection may have resulted in

positive terminals within the interpeduncular nucleus. But this does not limit the interpretation of the principal findings of the present study. The identification of transmitter markers in identified axon terminals requires electron microscopic double labelling. Here we visualized the incoming fibres mainly with peroxidase/DAB technique and used immunogold procedures to stain transmitters or corresponding markers. Unfortunately, the direct demonstration of GABA-like immunoreactivity via post-embedding staining requires a fixative with a high (>2.5%) glutaraldehyde concentration to keep all GABA molecules linked within the section. In our material a low (0.05%) glutaraldehyde concentration was necessary to preserve the immunoreactivity of marker proteins. Thus, direct visualization of GABA by post-embedding staining only yielded clear results at high concentrations as in presynaptic terminals (see Fig. 11D), while GABA-like immunoreactivity in dendrites remained equivocal (see Fig. 11C, D). Consequently, GABAergic cells and terminals were demonstrated with antibodies against glutamic acid decarboxylases. This enzyme is expressed in two isoforms of different molecular weights (65 or 67 kD). GAD65-immunoreactivity is more pronounced in axons and GAD67-protein more prominent in cell bodies and dendrites (Esclapez et al. 1994). Thus, probes and antibodies against both isoforms were used.

4.2. Projections from the LHb predominantly target GABAergic neurons in the ventral midbrain

In recent years considerable data have accumulated indicating that the LHb mostly exerts inhibitory influence on the activity of dopaminergic VTA neurons (see introduction). Our findings in the present report strongly suggest that the output from the LHb to the VTA and the RMTg in principle is excitatory but mostly targets inhibitory neurons. The VTA contains about 20% GABAergic neurons (Mugnaini and Oertel 1985; Kalivas 1993) and at least some of them are projection neurons (Van Bockstaele and Pickel 1995; Carr and Sesack 2000). The RMTg contains even more GABAergic cells projecting to the VTA (Geisler and Trimble 2008; Jhou et al. 2009a, b; Kaufling et al. 2009). The idea of GABAergic inhibition within the VTA or from the adjacent RMTg is in line with recent data. Thus, selective ablation of GABAergic neurons in the VTA increases the dopaminergic tone in the basal ganglia (Shank et al. 2007) and the direct electrical stimulation of the LHb yields an extended (about 80 msec) and largely complete inhibition of

dopaminergic activity in the VTA (Ji and Shepard 2007). Apparently, this inhibition is due to the converging inputs directly from the LHb and via a LHb-RMTg-VTA loop (Jhou et al. 2009a).

4.3. A small portion of LHb axons terminates on dopaminergic neurons in the VTA

A small number of LHb-derived axon terminals makes synapses with dopaminergic (tyrosin hydroxylase-positive) profiles in the VTA. While these data (Brinschwitz et al. 2010) first appeared surprising, recent electrophysiological experiments (Ji and Shepard 2007) have uncovered that a few dopaminergic VTA neurons indeed displayed excitatory responses to habenular stimulation. The highly complex subnuclear organization of the LHb has been appreciated quite recently (Andres et al. 1999; Geisler et al. 2003). New electrophysiological data support the idea of functional differences between the subnuclei (Kowski et al. 2009). Neurons with separate localizations in the LHb, therefore, may well provoke different effects in the VTA.

This idea is supported by the fact that lesioning the fasciculus retroflexus did not decrease the number of LHb-activated neurons in the VTA (Ji and Shepard 2007). Apparently, the axons of those cells in the LHb, which activate DA-neurons, do not use the fasciculus retroflexus on their way to the VTA.

4.4. Functional considerations

The mesocorticolimbic DA system, neurons in the VTA projecting throughout the limbic forebrain (Swanson 1982), is widely implicated in the mechanism of natural reward. It responds when the outcome of an action differs from the expected result (Schultz 1998). Set-point deviations at the unconscious level (increased or decreased body core temperature, plasma osmolality, or food consumption) most likely are sensed in the hypothalamus, while at the conscious level (successes or errors) the medial prefrontal cortex appears to be involved (Matsumoto et al. 2003; Ullsperger and von Cramon 2003). Detection of positive set-point deviations activates mesocorticolimbic DA neurons, resulting in pleasurable (hedonic) feelings, which are strongly correlated with an elevated

DA concentration in the nucleus accumbens (Blood and Zatorre 2001; Sabatinelli et al. 2007).

What had been missing is a structure, which converts negative set-point deviations to a reduced DA concentration. Recent data (Matsumoto and Hikosaka 2007) suggest that the LHb represents this “missing link”. Thus, we know that glutamatergic LHb projections excite inhibitory interneurons in the VTA (present data) and that selective removal of GABAergic interneurons in the VTA increases the dopaminergic tone in the striatum (Shank et al. 2007). Furthermore, electrical stimulation in the LHb largely decreases the activity of DA neurons in the VTA (Ji and Shepard 2007; Christoph et al. 1986), which extend their axons to the nucleus accumbens. Thus, the LHb is indeed able to control the DA concentration in the accumbens. This control may be achieved by a more or less tonic inhibition of DA neurons in the VTA (Nishikawa et al. 1986; Lecourtier et al. 2008). In line with this idea, abolishing the input from the LHb by bilateral infusion of tetrodotoxin into the fasciculus retroflexus or inhibiting the neuronal activity in the LHb by AMPA antagonists increases DA release and metabolism in striatal regions including the accumbens (Nishikawa et al. 1986; Lecourtier et al. 2008).

There is additional evidence to support the idea that the LHb represents the modulator converting set-point deviations to dopaminergic tone. The LHb receives a strong input from the preoptic area (Herkenham and Nauta 1977; Kowski et al. 2008) and the hypothalamus (Herkenham and Nauta 1977). This has to be expected if the LHb needs to be informed about set-point deviations at the basal homeostatic level. At the conscious level on the other hand, the LHb is the only structure strongly flashing up in functional magnetic resonance experiments, when the probands were informed about performance errors (Ullsperger and von Cramon 2003; Shepard et al. 2006). Most interestingly, such responses were not observed in schizophrenic individuals (Shepard et al. 2006). Numerous studies have further linked an overactivity of the LHb to the pathophysiology of depression (Winter et al. 2010; Caldecott-Hazard et al. 1988; Sartorius and Henn 2007; Morris et al. 1999).

In conclusion, we have shown that excitatory projections from the LHb predominantly target inhibitory interneurons in the VTA. Our results corroborate electrophysiological studies demonstrating an inhibitory effect of the LHb on the activity of dopaminergic neurons in the VTA. Activation of the LHb may therefore be an important mechanism for the control of dopamine release from the midbrain. Since a dysregulation of the communication between the two structures has been linked to the development of schizophrenia or depression, our results may provide a rationale for the development of innovative therapeutic strategies like deep brain stimulation of the LHb for the treatment of these diseases.

References:

1 Andres KH, von Düring M, Veh RW (1999): Subnuclear organization of the rat habenular complexes. J Comp Neurol. 407:130–150.

2 Araki M, McGeer PL, McGeer EG (1984): Retrograde HRP tracing combined with a pharmacohistochemical method for GABA transaminase for the identification of presumptive GABAergic projections to the habenula. Brain Res. 304(2):271-7.

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

4 Basbaum AI, Menetrey D (1987): WGA-apo-HRP-gold: a new retrograde tracer for light- and electron-microscopic single—and double-label studies. J Comp Neurol. 261:306–318.

5 Bayer VE, Pickel VM (1990): Ultrastructural localization of tyrosine hydroxylase in the rat ventral tegmental area: relationship between immunolabeling density and neuronal associations. J Neurosci. 10(9):2996-3013.

6 Bayer VE, Pickel VM (1991): GABA-labeled terminals form proportionally more synapses with dopaminergic neurons containing low densities of tyrosine hydroxylase-immunoreactivity in rat ventral tegmental area. Brain Res. 559(1):44-55.

7 Beckstead RM, Domesick VB, Nauta WJ (1979): Efferent connections of the substantia nigra and ventral tegmental area in the rat. Brain Res. 175(2):191-217.

8 Behzadi G, Kalén 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(1):77-100.

9 Blood AJ, Zatorre RJ (2001): Intensely pleasurable responses to music correlate with activity in brain regions implicated in reward and emotion. Proc Natl Acad Sci USA. 98:11818–11823.

10 Brinschwitz K, A. Dittgen, Madai V, Lommel R, Geisler S, Veh RW (2010): Glutamatergic fibers from the lateral habenula mainly terminate on GABAergic neurons of the ventral midbrain. Neuroscience. 168(2):463-76.

11 Bunney BS, Aghajanian GK (1976): Dopaminergic infllence in the basal ganglia: evidence for striatonigral feedback regulation. Res Publ Assoc Res Nerv Ment Dis. 55:249-67.

12 Caldecott-Hazard S and Phelps M (1988): Cerebral Correlates of Depressed Behavior in Rats, Visualized Using 14C-2-Deoxyglucose Autoradiography. J Neurosci. 8(6):1951-61.

13 Carr DB, Sesack SR (2000): GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. Synapse 38:114–123.

14 Chen BT, Rice ME (2002): Synaptic regulation of somatodendritic dopamine release by glutamate and GABA differs between substantia nigra and ventral tegmental area. J Neurochem. 81(1):158-69.

15 Christie MJ, Rowe PJ, Beart PM (1986): Effect of excitotoxin lesions in the medical prefrontal cortex on cortical and subcortical catecholamine turnover in the rat. J Neurochem. 47(5):1593-7.

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

17 Cohen SR, Melzack R (1993): The habenula and pain: repeated electrical stimulation produces prolonged analgesia but lesions have no effect on formalin pain or morphine analgesia. Behav Brain Res. 54(2):171-8.

18 Dittgen A, Gruber C, Geisler S, Zahm DS, Veh RW (2006): Anterior and posterior parts of the rat ventral tegmental area receive topographically distinct afferents from the lateral habenular complex. Soc Neurosci Abst Atlanta 670–624.

19 Ellison G (2002): Neural degeneration following chronic stimulant abuse reveals a weak link in brain, fasciculus retroflexus, implying the loss of forebrain control circuitry. Eur Neuropsychopharmacol. 12(4):287-97.

20 Ellison G (1994): Stimulant-induced psychosis, the dopamine theory of schizophrenia, and the habenula. Brain Res Brain Res Rev. 19(2):223-39.

21 Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR (1994): Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. J Neurosci. 14:1834–1855.

22 Felton TM, Linton L, Rosenblatt JS, Morell JI (1999): First and second order maternal behavior related afferents of the lateral habenula. Neuroreport. 10(4):883-7.

23 Forster GL, Blaha CD (2000): Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. Eur J Neurosci. 12:3596–3604.

24 Fuchs P, Cox VC (1993): Habenula lesions attenuate lateral hypothalamic analgesia in the formalin test. Neuroreport. 4(2):121-4.

25 Funk D, Stewart J (1992): The effects of lesions of the habenular nuclei on the development of sensitization to the behavioral activational effects of repeatedly administered morphine in the rat. Brain Res. 583(1-2):127-36.

26 Gariano RF, Groves PM (1988): Burst firing induced in midbrain dopamine neurons by stimulation of the medial prefrontal and anterior cingulate cortices. Brain Res. 462(1):194-8.

27 Garris PA, Ciolkowski EL, Pastore P, Wightman RM (1994): Efflux of dopamine from the synaptic cleft in the nucleus accumbens of the rat brain. J Neurosci. 14(10):6084-93.

28 Garris PA, Wightman RM (1995): Distinct pharmacological regulation of evoked dopamine efflux in the amygdala and striatum of the rat in vivo. Synapse. 20(3):269-79.

29 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.

30 Geisler S, Derst C, Veh RW, Zahm DS (2007): Glutamatergic afferents of the ventral tegmental area in the rat. J Neurosci. 27:5730–5743.

31 Geisler S, Trimble M (2008): The lateral habenula: no longer neglected. CNS Spectr. 13:484–489.

32 Geisler S, Zahm DS (2005): Afferents of the ventral tegmental are the rat - anatomical substratum for integrative functions. J Comp Neurol. 490:270–294.

33 Gonon FG (1988): Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. Neuroscience. 24(1):19-28.

34 Haun F, Eckenrode TC, Murray M (1992): Habenula and thalamus cell transplants restore normal sleep behaviors disrupted by denervation of the interpeduncular nucleus. J Neurosci. 12(8):3282-90.

35 Halliday GM, Törk I (1986): Comparative anatomy of the ventromedial mesencephalic tegmentum in the rat, cat, monkey and human. J Comp Neurol. 252(4):423-45.

36 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.

37 Herkenham M, Nauta WJ (1979): Efferent connections of the habenular nuclei in the rat. J Comp Neurol. 18:19–48.

38 Jhou T, Geisler S, Marinelli M, Degarmo BA, Zahm DS (2009a): 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.

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

40 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.

41 Johnson SW, Seutin V, North RA (1992): Burst firing in dopamine neurons induced by N-methyl-D-aspartate: role of electrogenic sodium pump. Science. 258:665–667.

42 Jongen-Rêlo AL, Amaral DG (2000): A double labeling technique using WGA-apoHRP-gold as a retrograde tracer and non-isotopic in situ hybridization histochemistry for the detection of mRNA. J Neurosci Methods. 101:9–17.

43 Kalivas PW (1993): Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. Brain Res Rev. 18:75–113.

44 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.

45 Kelly PH (1998): Defective inhibition of dream event memory formation: a hypothesized mechanism in the onset and progression of symptoms of schizophrenia. Brain Res Bull. 46(3):189-97.

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

47 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(4):1465-78.

48 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.

49 Lisoprawski A, Herve D, Blanc G, Glowinski J, Tassin JP (1980): Selective activation of the mesocortico-frontal dopaminergic neurons induced by lesions of the habenula in the rat. Brain Res. 183:229–234.

50 Lodge DJ, Grace AA (2006): The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. Proc Natl Acad Sci USA.

103:5167–5172.

51 Mahieux G, Benabid AL (1987): Naloxone-reversible analgesia induced by electrical stimulation of the habenula in the rat. Brain Res. 406(1-2):118-29.

52 Matsuda Y, Fujimura K (1992): Action of habenular efferents on ventral tegmental area neurons studied in vitro. Brain Res Bull. 28:743–749.

53 Matsumoto M, Hikosaka O (2007): Lateral habenula as a source of negative reward signals in dopamine neurons. Nature. 447:1111–1115. DOI:10.1038.

54 Matsumoto K, Suzuki W, Tanaka K (2003): Neuronal orrelates of goal-based motor selection in the prefrontal cortex. Science. 301:229–232.

55 Matthews-Felton T, Corodimas KP, Rosenblatt JS, Morrell JI (1995): Lateral-habenula neurons are necessary for the hormonal onset of maternal behavior and for the display of postpartum estrus in naturally parturient female rats. Behav Neurosci. 109(6):1172-88.

56 Morris JS, Smith KA, Cowen PJ, Friston KJ, Dolan RJ (1999): Covariation of activity in habenula and dorsal raphé nuclei following tryptophan depletion. Neuroimage. 10(2):163-72.

57 Mugnaini E, Oertel WH (1985): An atlas of the distribution of GABAergic neurons and terminals in the rat CNS as revealed by GAD immunohistochemistry.
In: Handbook of chemical neuroanatomy. Amsterdam: Elsevier.

58 Nagy JI, Carter DA, Lehmann J, Fibiger HC (1978): Evidence for a GABA-containing projection from the entopeduncular nucleus to the lateral habenula in the rat. Brain Res. 145(2):360-4.

59 Nauta HJ (1974): Evidence of a pallidohabenular pathway in the cat.
J Comp Neurol. 156(1):19-27.

60 Nishikawa T, Fage D, Scatton B (1986): Evidence for, and nature of the tonic inhibitory influence of habenulointerpeduncular pathways upon cerebral dopaminegic transmission in the rat. Brain Res. 373:324–336.

61 Oades RD, Halliday GM (1987): Ventral tegmental (A 10) system: neurobiology. 1. Anatomy and connectivity. Brain Res Rev. 12:117–165.

62 Paxinos G, Watson C (1998): The rat brain in stereotaxic coordinates.
San Diego: Academic Press.

64 Parent A. (1979): Identification of the pallidal and peripallidal cells projecting to the habenula in monkey. Neurosci Lett. 15(2-3):159-64.

65 Pasquier DA, Kemper TL, Forbes WB, Morgane PJ. (1977): Dorsal raphe, substantia nigra and locus coeruleus: interconnections with each other and the neostriatum. Brain Res Bull. 2(5):323-39.

66 Phillipson OT (1979): Afferent projections to the ventral tegmental area of tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. J Comp Neurol. 187:117–144.

67 Phillipson OT, Pycock CJ. (1982): Dopamine neurones of the ventral tegmentum project to both medial and lateral habenula. Some implications for habenular function. Exp Brain Res. 45(1-2):89-94.

68 Rajakumar N, Elisevich K, Flumerfelt BA. (1993): Compartmental origin of the striato-entopeduncular projection in the rat. J Comp Neurol. 331(2):286-96.

69 Redgrave P, Prescott TJ, Gurney K. (1999): Is the short-latency dopamine response too short to signal reward error? Trends Neurosci. 22(4):146-51.

70 Reynolds ES (1963): The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol. 17:208–212.

71 Sabatinelli D, Bradley MM, Lang PJ, Costa VD, Versace F (2007): Pleasure rather than salience activates human nucleus accumbens and medial prefrontal cortex. J Neurophysiol. 98:1374–1379.

72 Sandyk R. (1992): Pineal and habenula calcification in schizophrenia. Int J Neurosci. 67(1-4):19-30.

73 Sartorius A, Kiening KL, Kirsch P, von Gall C, 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(2):e9-e11.

74 Schultz W (1998): Predictive reward signal of dopamine neurons. J Neurophysiol. 80:1–27.

75 Sesack SR, Pickel VM. (1992): Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. J Comp Neurol. 320(2):145-60.

76 Sidibé M, Bevan MD, Bolam JP, Smith Y. (1997): Efferent connections of the internal globus pallidus in the squirrel monkey: I. Topography and synaptic organization of the pallidothalamic projection. J Comp Neurol. 382(3):323-47.

77 Skagerberg G, Lindvall O, Björklund A. (1984): Origin, course and termination of the mesohabenular dopamine pathway in the rat. Brain Res. 307(1-2):99-108.

78 Shank EJ, Seitz PK, Bubar MJ, Stutz SJ, Cunningham KA (2007): Selective ablation of GABA neurons in the ventral tegmental area increases spontaneous locomotor activity. Behav Neurosci. 121:1224–1233.

79 Shepard PD, Holcomb HH, Gold JM (2006): The presence of absence: habenular regulation of dopamine neurons and the encoding of negative outcomes. Schizophr Bull. 32:417–421.

80 Steffensen SC, Svingos AL, Pickel VM, Henriksen SJ. (1998): Electrophysiological characterization of GABAergic neurons in the ventral tegmental area. J Neurosci. 18(19):8003-15.

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

82 Swanson LW (1982): The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. Brain Res Bull. 9:321–353.

83 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.

84 Van Bockstaele EJ, Pickel VM (1995): GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain. Brain Res. 682:215–221.

85 Wagner CK, Silverman AJ, Morrell JI. (1998): Evidence for estrogen receptor in cell nuclei and axon terminals within the lateral habenula of the rat: regulation during pregnancy. J Comp Neurol. 392(3):330-42.

86 Winter C, Vollmayr B, Djodari-Irani A, J. Klein J, Sartorius A (2010): Pharmacological inhibition of the lateral habenula improves depressive-like behavior in an animal model of treatment resistant depression. Behav Brain Res. (epub ahead of print)

87 Wu M, Hrycyshyn AW, Brudzynski SM (1996): Subpallidal outputs to the nucleus accumbens and the ventral tegmental area: anatomical and electrophysiological studies. Brain Res. 740:151–161.

88 Zahm DS, Heimer L (1990): Two transpallidal pathways originating in the rat nucleus accumbens. J Comp Neurol. 302:437–446.

Acknowledgments

Diese Arbeit wäre ohne die Unterstützung und Motivation vieler, wichtiger Menschen nicht möglich gewesen. Zu aller erst möchte ich meinen Eltern für ihren ausdauernden Antrieb und ihr Vertrauen danken. Die Frage “Und, was macht Deine Doktorarbeit?” hört man doch immer gern. Vielen Dank Anja für die schöne Zeit im Labor und die erfolgreichen Ablenkungsmanöver. Ganz besonderen Dank geht an Heike, Petra und Ina ohne deren Unterstützung diese Arbeit nicht möglich gewesen wäre. Ein großes Dankeschön geht an Gregor Laube, der bei Problemen bei der Elektronenmikroskopie immer zur Stelle war und mich regelmäßig mit hochkalorischen Nahrungsmitteln versorgt hat. Gedankt seien René Bernard und Frau Schulte für ihre Kritik an meinem Manuskript. Ein weiterer Dank geht an Annett, die mein allgemeines Chaos immer wieder geordnet hat.

Ohne Alex wäre es wohl nie zur Vollendung der Promotion gekommen. Vielen Dank für deine uneingeschränkte Unterstützung. Du bist großartig.

Zuletzt möchte ich mich bei meinem Doktorvater Prof. Veh für die Überlassung des interessanten Themas und die stete Unterstützung bedanken.

Erklärung

“Ich, Katja Brinschwitz, erkläre hiermit, dass ich die vorgelegte Dissertationsschrift mit dem Thema “Glutamatergic axons from the lateral habenula mainly terminate on GABAergic neurons in the VTA” selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzuläßige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.”

27.09.2010

Datum Unterschrift

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