Refinement of the dopaminergic system of anuran amphibians based on connectivity with habenula, basal ganglia, limbic system, pallium, and spinal cord

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Abstract

Whereas our understanding of the dopaminergic system in mammals allows for a distinction between ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), no clear evidence for separate structures in anamniotes has been presented to date. To broaden the insight into the organization and regulation of neuromodulatory systems in anuran amphibians, tracing and immunohistochemical investigations were performed in the Oriental fire-bellied toad, Bombina orientalis. Topographically organized catecholaminergic "nigrostriatal," "mesolimbic," "mesocortical," and spinal cord projections arising from the posterior tubercle and mesencephalic tegmentum were identified. We compared these results with published data from lampreys, chondrichthyes, teleosts, amphibians, reptiles, birds, and mammals. Based on the pattern of organization, as well as the differential innervation by the habenular nuclei, domains gradually comparable to the mammalian parangral VTA, ventral tier of the SNc, interfascicular nucleus of the VTA, and supramamillary/retromamillary area were identified. Additionally, we could demonstrate topographic separate populations of habenula neurons projecting via a direct excitatory or indirect GABAergic pathway onto the catecholaminergic VTA/SNc homologs and serotonergic raphe nuclei. The indirect GABAergic habenula pathway derives from neurons in the superficial mamillary area, which in terms of its connectivity and chemoarchitecture resembles the mammalian rostromedial tegmental nucleus. These results demonstrate a much more elaborate interconnection principle of the anuran dopaminergic system than...
INTRODUCTION

In mammals, dopaminergic neurons can be separated into 17 groups (designated A1–A17). The A9 cell group is for the most part identical with the substantia nigra pars compacta (SNc). A10 neurons are mostly located in the ventral tegmental area (VTA; Björklund & Dunnett, 2007) whereas dopaminergic cells in A11 are found (depending on the species) in the posterior periventricular nucleus (Felten & Sladek, 1983), hypothalamus and supramamillary/retromamillary area (Björklund & Dunnett, 2007). Studies of the connections, chemoarchitecture, and development of the amphibian brain have revealed a comparable pattern of organization. This includes neurons in the dorsomedial posterior tubercle (i.e., SNc) and neurons in the basal synencephalic plate and ventral tegumentum (i.e., VTA). But the anuran A9–A10 complex is more compact than the corresponding mammalian structures and lacks the laterally migrated substantia nigra. In contrast, the A9 and A10 cell groups of birds and reptiles seem to exhibit a higher degree of topographic separation (reviewed by Smeets & González, 2000; Smeets, Marín, & González, 2000). The amphibian homologs of the A9–A10 cell groups might therefore represent an early stage in the evolution of these structures. However, in a recent study we could identify a differential innervation of the SNc/VTA homologs by the ventral and dorsal habenula in Bombina orientalis (Freudenmacher, Twickel, & Walkowiak, 2019). This closely resembles the situation found in many mammals (Akagi & Powell, 1968; Araki, McGeer, & Kimura, 1988; Brinschwitz et al., 2010; Herkenham & Nauta, 1979; Omelchenko, Bell, & Sesack, 2009; Phillipson, 1979; Way & Kaelber, 1969). The in-depth analysis of the habenula circuitry in anuran amphibians could therefore provide new insights into the evolution of dopaminergic systems. In all mammals for which data are available, the habenula comprises medial (MHb) and lateral parts (LHb; reviewed by Hikosaka, 2010), which correspond—in terms of their connectivity—to the dorsal (DHb) and ventral habenula (VHb) of anuran amphibians (Freudenmacher et al., 2019).

The habenula takes part in regulating the activity of neuromodulatory neurons via direct glutamatergic and indirect GABAergic pathways, a feature conserved from lampreys (Stephenson-Jones, Floros, Robertson, & Grillner, 2012) to mammals (Brown & Shepard, 2016). In rodents, effenter habenula projections onto GABAergic, dopaminergic, and serotonergic brain regions originate from topographic distinct populations within the habenula subnuclei (Bernard & Veh, 2012; Gonçalves, Sego, & Metzger, 2012). The stimulation of the LHb in rodents leads to the inhibition of dopaminergic neurons in the VTA and SNc (Christoph, Leonzio, & Wilcox, 1986) and serotonergic neurons in the raphe nuclei (Wang & Aghajanian, 1977). This appears to be mediated by GABAergic neurons in the rostromedial tegmental nucleus (RMTg) and opposes the reward and motor-activating functions of dopaminergic midbrain neurons (Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009). While a structure homologous to the RMTg—located in the dorsal mamillary area—was identified in lampreys (Stephenson-Jones et al., 2012), we could identify the superficial mamillary area as a putative RMTg homolog in anuran amphibians (Freudenmacher et al., 2019). In order to address the question whether the MHb/LHb–RMTg–VTA/SNc/raphe circuitry is as well present in anuran amphibians, this study investigated these regions and their topographic and immunohistochemical organization in more detail. Investigations of the anuran habenula circuitry together with immunohistochemical analysis of "nigrostriatal," "mesolimbic" and spinal cord projections, as well as a hypothesized "mesocortical" projection, could provide additional insight to which extend the anuran SNc–VTA can be regarded to reflect the ancestral state of tetrapods. Consequently, an attempt was made to refine the neuroanatomical framework of the dopaminergic circuitry in anuran amphibians.

METHODS

2.1 Isolated brain preparations

Subject of investigation was the isolated brain of the oriental firebelled toad, Bombina orientalis. Fifty-three adult animals of either sex, taken from our own breeding colony or purchased from an animal supplier (Hoch, Waldkirch, Germany), were used in the present study. The animals were deeply anesthetized by immersion in 0.2% (wt/vol) tricane methanesulfonate (MS-222, Sigma–Aldrich, Cat# E10521) in tap water for 10 min (Luksch, Walkowiak, Muñoz, & ten Donkelaar, 1996; Ohr, 1976). The body temperature was cooled down on ice, the medulla severed with a surgical chisel and the head removed. The brains were isolated by a ventral approach and transferred into a dish with Ringer’s solution (75 mM NaCl, 25 mM NaHCO₃, 2 mM CaCl₂, 2 mM KCl, 0.5 mM MgCl₂, 11 mM D-glucose), which was adjusted...
with carbogen (95% O₂, 5% CO₂) to a pH of 7.4 and stored at 5°C. The majority of the brain preparations were also used in a prior study (Freudenmacher et al., 2019). The project is registered after §4 of the Animal Protection Act under the title "NeuroAnura," approval number 4.16.004.

2.2 | Anatomical tracings

For the anatomical reconstruction of the habenula and neuromodulatory circuitries, small deposits of neuronal tracers were placed in various brain areas. The neuronal tracer Neurobiotin [Vector Laboratories, Cat# SP-1120; 20% (wt/vol) in distilled water] was pressure-injected using glass micropipettes into various brain areas in whole brain or half brain preparations (transacted midsagittally). The locations of tracer applications are shown in Table 1. Tracer application to the medulla oblongata was achieved by immersion of the severed tissue in 20% Neurobiotin solution (wt/vol) for 15 min. After the tracer applications, the brains were kept in Ringer’s solution for 24–48 hr at 5°C to allow anterograde and retrograde transport. Ringer’s solution was changed at regular intervals to ensure a stable pH and oxygen supply.

### Table 1 Tract and cell tracing experiments conducted to unravel the dopaminergic circuitry

| Injection site | No. of experiments, hemisphere | Type of brain preparation |
|----------------|-------------------------------|---------------------------|
| Hb             | (5) left, (5) right           | Whole and half brain      |
| VHb            | (4) left, (2) right           | Whole and half brain      |
| DHb            | (2) right, (1) left           | Whole and half brain      |
| Acc            | (3) left                      | Whole brain               |
| Str            | (2) right, (2) left           | Whole brain               |
| TPdm           | (6) medial                    | Whole brain or partly removed ventral infundibulum |
| TPdm/Rm        | (2) right, (2) left in N = 2  | Half brain or partly removed ventral infundibulum |
| IF             | (4) medial                    | Whole brain               |
| IP             | (3) medial                    | Whole brain               |
| RaM (level: Cerebellum) | (3) medial            | Whole brain               |
| RaM (level: Superior olive) | (2) medial         | Whole brain               |
| Sm             | (3) right, (3) left           | Whole and half brain      |
| med            | (3) bilateral                 | Whole brain               |

Note: Total number of experimental animals: 53. Abbreviations: Acc, nucleus accumbens; DHb, dorsal habenula; Hb, habenula; IF, interfacular nucleus of the ventral tegmental area; IP, interpeduncular nucleus; med, medulla oblongata; RaM, nucleus raphe magnus; Sm, superficial mamillary area; Str, striatum; TPdm, dorsomedial posterior tubercle; TPdm/Rm, dorsomedial posterior tubercle and retromammillary area; VHb, ventral habenula.

2.3 | Immunohistochemical procedures

Fixation of the brain tissue was achieved by immersion overnight at 5°C in a phosphate- or tris-buffered fixative (for details see Table 2). Subsequently, the brains were cryoprotected for 3–12 hr with 20% (wt/vol) sucrose in phosphate buffer (PB; +1% sodium metabisulfite for histamine stainings), embedded in Tissue-Tek mounting medium (Sakura, Cat# 4583) and rapidly frozen. Transverse sections (20–30 μm) were produced with a cryostat (Leica CM 3050 S). The sections were collected in two series on adhesion slides (Superfrost Plus, Thermo Scientific) and stored at –20°C until processing. Prior to further treatment, the sections were dried for up to 2 hr at 37°C. Heat induced epitope retrieval (HIER) was used for GABA detection (8 min at 80°C in 10 mM sodium citrate, pH 9). All antibodies were obtained commercially, the specificity of the affinity-purified antibodies used was assessed by the suppliers (see Table 2). The specificity of the tyrosine hydroxylase (TH) antibody was previously assessed in a wide range of vertebrate species, in particular in anuran and urodele amphibians (Domínguez, Morona, González, & Moreno, 2013; Morona & González, 2008). The slices were incubated overnight at room temperature (RT) with the primary antibody (see Table 2). The slices were rinsed with phosphate-buffered saline [PBS; or tris-buffered saline (TBS) for histamine stainings] and incubated with 1:500 secondary antibody (see Table 2), 1:500 deep-red fluorescent Nissl stain (Molecular Probes, Cat# N21483, RRID:AB_2572212) and 1:100 streptavidin conjugated with Alexa Fluor 488 or Cy3 (Jackson ImmunoResearch, Cat# 016-540-084, RRID:AB_2337249; Cat# 016-160-084, RRID:AB_2337244) in 1% (wt/vol) bovine serum albumin [BSA; in 0.3% (vol/vol) Triton-X 100 in 0.1 M PB; or 0.2% (vol/vol) Triton-X 100, 1% sodium metabisulfite in 0.05 M TBS for histamine stainings]. The slices were washed with PBS (or TBS for histamine stainings) and covered with glycerol containing 2.5% (vol/vol) DABCO (diazabicyclonooctane) and a coverslip. In control experiments, either the primary or the secondary antibody was omitted.

2.4 | Analysis of histological stainings

The sections were examined with a confocal laser scanning microscope (Zeiss LSM 510). Images of injection sites were partly taken with a fluorescence microscope (coupled to a digital camera, Zeiss AxioCam HR). The specificity of the antibodies was compared with previous studies. These demonstrated that the majority of tyrosine hydroxylase immunoactive (THir) neurons in the posterior tubercle/ventral tegmentum are dopaminergic. The classification of the dopaminergic/catecholaminergic system is therefore based on González and Smeets (1991), Smeets et al. (2000), O’Connell, Matthews, Ryan, and Hofmann (2010), Dominguez, González, and Moreno (2014), and own conclusions (see discussion). The serotonergic raphe nuclei were identified after Adli, Stuesse, and Cruce (1999) and Stuesse, Adli, and Cruce (2001) and the histaminergic areas were classified after Airaksinen and Panula (1990). Two-dimensional projections were created out of three-dimensional z-stacks using ImageJ (Version 1.51s; RRID: SCR_003070). Reconstructions of THir neurons were made by hand...
with the aid of a camera lucida. Figures were generated using Adobe Illustrator (Adobe Illustrator CS5, version 15.0.0, RRID:SCR_010279).

3 | RESULTS

Based on direct and complementary injections, the afferent and efferent connectivity of the habenular nuclei, superficial mamillary area, and the dopaminergic and serotonergic system was identified. Application of Neurobiotin into the DHb and VHb resulted in the labeling of the fasciculus retroflexus (fr). This main efferent path is composed of a DHb pathway—consisting of the habenulo-interpeduncular (hbip) and habenulo-interfascicular-tract (hbif)—and a VHb pathway—consisting of the habenulo-posterior-tubercle (hbpt), habenulo-superficial-mamillar (hbsm), and habenulo-raphe tract (hbra). Since the topographically differential innervation by habenula neurons is a decisive argument for the refinement of the anuran SNc–VTA complex, connections to the interpeduncular nucleus and raphe nuclei were assessed to exclude fibers of passage. Few references were included in the result section to place these findings in the context of recent anatomical classifications.

3.1 | Topographic organization of the habenular nuclei

The connectivity and topographic organization of the right dorsal (r. DHb), right ventral (r.VHb), left dorsomedial (l.DmHb), left dorsolateral (l.DlHb), and left ventral (l.VHb) habenular nuclei were investigated by tracer injections into the superficial mamillary area (Sm), dorsomedial posterior tubercle (TPdm), interfascicular nucleus of the ventral tegmental area (IF), interpeduncular nucleus (IP), and raphe nuclei. The different habenula subnuclei are illustrated in Figure 1. Each habenula subnucleus is a hollow structure, in which densely packed neurons surround the relative cell-poor core. Because of its medial and lateral components, the left dorsal habenula is considerably larger at its rostral end but does not extend further in the rostrocaudal axis (Figure 2). The l.DmHb is bigger than its lateral counterpart (l.DlHb) and unaccompanied at caudal levels. It also contains fewer free cells in its core than the nucleus on the right site, possibly because of a partial habenula septum (p.s) innervating the core at rostral and intermediate levels.

3.2 | Superficial mamillary area projecting ventral habenula neurons

Retrograde labeling resulting from unilateral injections into the superficial mamillary area (Sm; n = 6) is documented in column (a) of Figure 2. Marked neurons were predominantly found ipsilateral in the rostral end but does not extend further in the rostrocaudal axis (Figure 2). The l.DmHb is bigger than its lateral counterpart (l.DlHb) and unaccompanied at caudal levels. It also contains fewer free cells in its core than the nucleus on the right site, possibly because of a partial habenula septum (p.s) innervating the core at rostral and intermediate levels.

3.3 | Dorsomedial and dorsolateral posterior tubercle projecting ventral habenula neurons

Small applications into the dorsomedial (TPdm), dorsolateral (TPdl), and the medial proportion of the retromamillary area (Rm) led to retrogradely labeled cells in the VHb core. Especially dense labeling was

| TABLE 2 | List of antibodies, dilutions, and treatment |
|----------|-----------------------------------------------|
| Primary antibody | Dilution | Fixation method | Secondary antibody |
| Rabbit anti-TH | 1:200 in 1% (wt/vol) BSA [0.3% Triton-X 100 in 0.1 M PB] | 4% PFA, 14% saturated TNP in 0.1PB | Goat anti-rabbit (Cy3 or Alexa Fluor 488, Jackson ImmunoResearch Labs, Cat# 111-165-144, RRID: AB_2338006; Cat# 111-545-144, RRID: AB_2338052) |
| Rabbit anti-GABA | 1:1000 in 1% (wt/vol) BSA [0.3% Triton-X 100 in 0.1 M PB] | 4% PFA, 14% saturated TNP, 0.5% GA in 0.1 PB | Goat anti-rabbit (Cy3 or Alexa Fluor 488, Jackson ImmunoResearch Labs) |
| Rabbit anti-5HT | 1:100 in 1% (wt/vol) BSA [0.3% Triton-X 100 in 0.1 M PB] | 4% PFA, 14% saturated TNP in 0.1 PB | Goat anti-rabbit (Cy3 or Alexa Fluor 488, Jackson ImmunoResearch Labs) |
| Mouse anti-histamine | 1:1000 in 1% (wt/vol) BSA [0.2% Triton-X 100, 1% sodium metabisulfite in 0.05 M TBS] | 4% (wt/vol) EDAC (Sigma–Aldrich, Cat# E7750) in 0.1 M PB. | Goat anti-mouse (Alexa Fluor 488, Jackson ImmunoResearch Labs, Cat# 115-545-146, RRID: AB_2307324) |

Abbreviations: BSA, bovine serum albumin; EDAC, 1-ethyl-3-(3-dimethylamino-propyl)-carboodimide hydrochloride; GA, glutaraldehyde; PB, phosphate buffer; PFA, paraformaldehyde; TBS, tris-buffered saline; TNP, picrinic acid.
also found in the ventromedial VHB wall (Figure 2: column b; \( n = 6 \)). Scattered axons of the fasciculus retroflexus can be found coursing through the anterior thalamic nucleus and along the third ventricle. Much larger injections into the Rm, TPdm, and TPdl by a lateral approach resulted in more abundant cell labeling in the VHB \(( n = 4 \) in \( N = 2 \)) but colabeling of the hbsm cannot be excluded.

### 3.4 | Interfascicular nucleus projecting dorsal habenula neurons

Furthermore, injection sites centered in the IF (Figure 2: column c; \( n = 4 \)) resulted in backfilled neurons exclusively \(( n = 2 \) or predominately \(( n = 2 \)) in the LDMHb and r.DHB. Most cells were found in the wall of the caudal DHB, their axons and dendrites extending into the inner core. Due to the lateral course of the hbit, these neurons can be assigned to a connection to the IF. Few scattered neurons in the VHB \(( n = 2 \)) and one single neuron in the LDIHb \(( n = 1 \)) can be attributed to fibers of passage.

### 3.5 | Interpeduncular nucleus projecting dorsal habenula neurons

Following tracer injections into the IP (Figure 2: column d; \( n = 3 \)), labeled neurons were found throughout the entire dorsal habenula. Labeling was denser at the caudal pole \(( n = 3 \)) and included marked cells in the DHB wall and free cells in the core. In contrast to the injections into the IF, backfilled cells were also found in the LDIHb. Few labeled neurons in the VHB can possibly be attributed to labeling of the hbra and to connections to the superior central and dorsal raphe (RaCS-RaD) complex, located dorsally to the IP.

### 3.6 | Raphe nuclei projecting ventral habenula neurons

Neurobiotin application into the nucleus raphe magnus (RaM) at the level of the cerebellum (Figure 2: column e; \( n = 3 \)) labeled neurons in the entire VHB, most of them in the inner core. The majority of marked neurons were found at intermediate and rostral levels. Most axons leave at the rostral pole and because of the particularly prominent labeling, it can be assumed that the hbra makes up a substantial proportion of the fr. The hbsm and hftp in comparison seems to follow a more distributed course. Labeled neurons in the DHB \(( n = 2 \)) can be explained by unintentional spread of the tracer along the descending tracks, because injections into the RaM at the level of the superior olive \(( n = 2 \)) led to few labeled neurons exclusively in the VHB.

Overall, habenular projections in Bombina orientalis are organized in a strikingly topographic manner.
3.7 | Habenula efferents to dopaminergic neurons

To determine if habenula fibers directly contact dopaminergic neurons, anterograde labeling was combined with immunohistochemistry. Tyrosine hydroxylase (TH) antibodies were used in the present study to reveal putative dopaminergic cell groups. In the caudal part of the diencephalon, partly continuous groups of TH positive neurons could be observed (Figure 3).

3.8 | Habenula efferents to the retromamillary area

Centrally located, TH immunoreactive (THir) cells were situated in the wall of the zona incerta of the periventricular nucleus (Zip). Some cells have apical processes, that protrude toward the ventricle, thus contacting the cerebrospinal fluid. The axons of these neurons radiate laterally into the diencephalon. Depending on the orientation of the axis of transverse sections, a crescent shaped THir cell group ventral or ventrocaudal to the Zip was observed. This group corresponds to the retromamillary area (Rm) as described by López, Morona, and González (2010) and was previously included in the rostral posterior tubercle (González, Marín, Tuinhof, & Smeets, 1994; González & Smeets, 1991). Anterograde labeled fibers from the left (N = 2) and right (N = 3) VHb were observed in close apposition to the THir cell bodies and dendrites (Figure 3a).

3.9 | Ventral habenula efferents to the dorsolateral posterior tubercle

Further, caudally, a single lateral extending THir cell layer can be observed. At its rostral pole it forms a ribbon like structure and
seems not to be affiliated with the hypothalamic mamillary area (Mam). In this study this cell group is referred to as the dorsolateral nucleus of the posterior tubercle (TPdl). A distinct plexus of VHb fibers aggregates around this area (hbtp). Terminal-like varicosities were found in close apposition to THir processes, cell bodies and near TH negative neurons (Figure 3b; left n = 2, right n = 3). These fibers are also relatively dense in comparison to axons innervating the retromamillary area. Ventrally located THir cells in the mamillary area accumulate in the deep densely packed layers and possess axons extending in a more ventral direction.

3.10 | Ventral habenula efferents to the dorsomedial posterior tubercle

A single group of THir neurons is found at the intermediate level of the posterior tubercle, consisting of small, round cells in its dorsomedial part (TPdm). They can be found between the floor of the third ventricle and the funnel-shaped infundibular recess (IR). The axons of this cell group radiate in a laterorostral direction. Neurons of the TPdl attach lateral to the TPdm and are in prior studies attributed to the TPdm. The lateral cells are slightly larger, pear-shaped, and their processes are predominantly oriented in a dorsolateral direction.
Retrogradely labeled cells in the TPdm/dl were only found following injections including the VHb (n = 4; data not shown). Located ventrally, the retromamillary area (Rm) joins the mamillary area (Mam) as a superficial loosely packed layer. This layer is divided from the TPdm/TPdl by the commissure of the posterior tubercle (ctp). At this point, the Rm, TPdm, and TPdl receive habenular input. The hbtn passes through the TPdm and few axons cross to the contralateral hemisphere where they terminate close to tyrosine hydroxylase positive neurons (Figure 3c; left n = 3, right n = 3). These THir groups disappear in the caudal direction, while THir neurons in the Rm can still be found.

3.11 | Dorsal habenula efferents to the interfascicular nucleus

At the level of the basal syncephalic plate (bs), a densely packed unpaired cell group is found dorsal to the caudal extend of the dorsomedial posterior tubercle (TPc). This unpaired cell group extends caudally as far as the roots of the oculomotor nerve. In contrast to the TPdm/TPdl, this cell formation receives only input from the dorsal habenula. In the descriptive sense of lying between the fascicles (fasciculus retroflexus) and in reference to the connectivity of mammals, this area is designated as the interfascicular nucleus of the ventral tegmental area (IF). Tracer application limited exclusively to the DHb led to the labeling of the thin dorsal habenulo-interfascicular tract. Terminal-like structures were found in close apposition to TH immunoreactive neurons and dendrites (Figure 3d; n = 3). Injections involving the DHb (n = 4) and in few cases the VHb (n = 2) lead to scattered labeled neurons in the IF. The habenulo-interpeduncular tract takes a further lateral course. The habenulo-raphe tract is not stained because of its VHb origin (Figure 3d).

3.12 | Ventral habenula efferents to histaminergic and serotonergic neurons

Following VHb tracer injection, thin axons run through the mamillary/retromamillary area, selectively targeting histaminergic cerebrofluid contacting cells (Hist) near the periventricular nucleus (white flat arrowheads, Figure 4a; right n = 2). Serotonergic (5HT) neurons can be found in the periventricular nucleus (Npv) and in a round dorsolateral migrated nucleus. Both the ventral as well as the dorsal Npv receive habenular input (Figure 4b; n = 2).

Injections into the VHb also resulted in labeling of the hbra. Few axons of this tract diverge into the superior central and dorsal raphe (RaCS-RaD) complex (Figure 4c; n = 3). The RaCS-RaD extends from the interfascicular nucleus to the level of the caudal interpeduncular nucleus. Proceeding caudally, the hbra extends to the nucleus raphe magnus (RaM). The raphe nucleus begins at level of the cerebellum and continues to the level of the superior olive in the medulla oblongata. The nucleus exhibits two symmetrical serotonergic cell rows with ventrally projecting dendrites. Few axons terminate close to serotonergic cell bodies, while the main tract concentrates ventrally, presumably innervating the serotonergic cell processes (Figure 4d; n = 3).

3.13 | Connections of the superficial mamillary area

The following section provides evidence for an indirect GABAergic pathway, that links the VHb to dopaminergic and serotonergic brain regions. This input derives from GABAergic neurons in the superficial mamillary area. Particularly strongly stained GABAergic neurons appear as the ventrolateral extension of the Rm (Figure 5a). Weaker stained neurons form an oval shaped nucleus, although the exact demarcation cannot be fully determined. Injections involving the VHb

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**Figure 4** Direct VHb projections to histaminergic and serotonergic neurons. Anterogradely labeled fibers following Neurobiotin injections (green) in (a, d) the VHb, and in (b, c) r.DHb/r.VHb in close vicinity of (a) histaminergic and (b–d) serotonergic neurons. Yellow arrowheads indicate labeled fibers close to immunoreactive neurons, white flat arrowheads liquor contacting neurons, white arrowheads anterogradely labeled fibers, white stars immunoreactive neurons. Microscopic pictures of the injection sites are shown in the bottom right corner. For abbreviations see list. Scale bars 25 μm [Color figure can be viewed at wileyonlinelibrary.com]
led to labeling of the hbsm, their axons finally terminating close to GABA immunoreactive cell bodies (Figure 5a; left \( n = 3 \), right \( n = 2 \)). To determine if a homolog of the mammalian RMTg is indeed present, the efferent structures were analyzed. Anterogradely labeled fibers from the superficial mamillary area were observed in close apposition to THir processes and cell bodies in the medial Rm, TPdm, and TPdl, but never IF. Additionally, retrogradely double-labeled cells in the TPdm could be found. Labeled cells in the mamillary/retromamillary area could not be confirmed to be THir in origin (Figure 5b; \( n = 3 \)). To test if this connection is GABAergic, complementary injections into the TPdm/dl were performed. In agreement with the described connections, GABAergic neurons in the superficial mamillary area were retrogradely labeled (Figure 5c; \( n = 2 \)). Most labeled cells are located ventral to the strongly GABAergic tail of the Rm. Neurobiotin application to the nucleus raphe magnus resulted in more dorsally located retrogradely labeled GABAergic neurons (Figure 5d; \( n = 3 \)). Furthermore, labeled axons innervating the migrated GABAergic cell band could be noticed. Finally, axons originating from the Sm can be found terminating near serotonergic cell bodies in the RaCS-RaD (Figure 5e) and RaM (Figure 5f). The axons follow a more scattered course and are not ventrally concentrated like the hbra. No anterogradely labeled fibers in the IF after Sm injection could be found.

In brief, these results demonstrate for the first time direct habenular projections to GABAergic and neuromodulatory neurons in anuran amphibians. This GABAergic region shares many additional characteristics with the mammalian RMTg, as summarized in the discussion.

3.14 | Dopaminergic projections of the posterior tubercle and interfascicular nucleus to the striatum, nucleus accumbens, rostral pallium, and medulla oblongata

Additional retrograde tracing experiments were performed to define the boundaries of the presumed anuran VTA and SNc homologs. Following injections of Neurobiotin in the nucleus accumbens, THir and Neurobiotin double labeled cells were found in the rostral and intermediate TPdl. Proceeding in caudal direction, few THir Neurobiotin double labeled neurons were found in the caudal extend of the posterior tubercle (TPc) and in the IF (Figure 6a–d; \( n = 3 \)). A differential...
distribution of THir nucleus accumbens projecting neurons could not be determined. The dopaminergic "mesolimbic" pathway originates therefore mainly from cells in the TPdl and IF. Furthermore, injections into the rostral pallium (rP) revealed retrogradely labeled THir neurons in the rostral and intermediate TPdl (Figure 6e; \( n = 2 \) in \( N = 3 \)), as well as in additional labeled TH negative cells (\( n = 3 \) in \( N = 3 \)). A dopaminergic "mesocortical" projection seems therefore to be present. Tracer application into the striatum led to few THir and Neurobiotin double-labeled cells located exclusively in the ventral TPdm (Figure 6f; \( n = 2 \)), as well as in additional labeled TH negative cells (\( n = 4 \) in \( N = 4 \)), representing the "nigrostriatal" pathway. In case of medulla oblongata applications (\( n = 3 \)) labeled THir neurons were found throughout the rostral TPdl and the Rm (Figure 6g,h; \( n = 3 \)).

4 | DISCUSSION

4.1 | Differential habenula projections

In a previous study, we identified prominent projections from the dorsal and ventral habenula to the posterior tubercle, mesencephalic tegmentum, and raphe nuclei in Bombina orientalis (Freudenmacher et al., 2019). But direct habenula projections to dopaminergic, serotonergic, and histaminergic neurons were not identified. The present study focused on these connections and demonstrated for the first time in anuran amphibians topographic distinct habenula subpopulations projecting via a direct excitatory or indirect GABAergic pathway onto these neuromodulatory neurons (Figure 7). Besides providing further arguments for the evolutionary conservation of the habenula circuitry in vertebrates, we oppose the conclusions of Laberge and Smith (2017) who consider the preservation of this system to be overrated. These results provide the basis for new insights into the topographic organization of the dopaminergic system of anuran amphibians. In rats, inputs to the RMTg mostly arise from the lateral division of the LHb, whereas inputs to the VTA mainly emerge from the medial division of the LHb (Gonçalves et al., 2012). Additionally, raphe nuclei and VTA projecting neurons are differently distributed within the LHb with respect to their rostrocaudal position (Bernard & Veh, 2012). In Bombina orientalis habenular projections are as well organized in a topographic manner. Inputs to the Sm arise mainly from the dorsolateral wall of the VHB, whereas inputs to the TPdm/TPdl emerged from the ventromedial part of the VHB. RaM efferents originate from free cells in the VHB. The r.DHb and l.DmHb give rise to IF projecting neurons, while IP output emerges additionally from free cells in the core and l.DIHb. This topographical analysis enabled us to exclude for the most part fibers of passage. One clear difference in the lamprey is that the homolog of the LHB is completely lateralized (Stephenson-Jones et al., 2019).

FIGURE 6 The anuran TP and IF is topographically organized. Tracer application to (a–d) the nucleus accumbens, (e) rostral pallium, (f) striatum and (g, h) medulla oblongata, lead to THir Neurobiotin double labeled neurons in (a, e, g) the rostral dorsolateral, (b) intermediate dorsolateral, (f) dorsomedial nucleus of the posterior tubercle, (h) retromamillary area, (c) caudal posterior tubercle, and (d) the interfascicular nucleus of the ventral tegmental area. Yellow arrowheads indicate THir and Neurobiotin double labeled neurons. For abbreviations see list. Scale bars 25 μm [Color figure can be viewed at wileyonlinelibrary.com]
et al., 2012), but a topographical organization is also present. Despite few differences, a common pattern in lampreys, anurans, and mammals can be determined.

### 4.2 The anuran VTA and SNc

In mammals, the precise temporal and spatial release of neuromodulators within the basal ganglia, limbic system, and cerebral cortex is essential for motor control and cognitive functions, as demonstrated by severe neurological deficits following pathological changes in dopamine levels (e.g., Parkinson’s disease; Sveinbjörnsdóttir, 2016). Comparably, the depletion of dopamine in the brain of ancient vertebrates (i.e., lampreys) and early tetrapods (i.e., anuran amphibians) leads to impaired motor behavior (Endepols, Schul, Gerhardt, & Walkowiak, 2004; Thompson, Ménard, Pombal, & Grillner, 2008) and, in anurans, also to “cognitive” symptoms, characteristic for human Parkinson patients (Endepols et al., 2004). In all anamniotes for which data are available, a dopaminergic/catecholaminergic cell group is located between the diencephalon and mesencephalon in the posterior tubercle. In many species these neurons give rise to dopaminergic projection to the basal forebrain [lamprey: Pérez-Fernández, Stephenson-Jones, Suryanarayana, Robertson, & Grillner, 2014, shark: Carrera, Anadon, & Rodriguez-Moldes, 2012; Quintana-Urzainqui et al., 2012, zebrafish: Rink & Wullimann, 2001, commentary by Wullimann, 2014]. Additionally, in some chondrichthyes and lungfishes a mesencephalic dopaminergic cell group is reported, but detailed information about the connectivity is not available (Meredith & Smeets, 1987; Northcutt, Reiner, & Karten, 1988; Reiner & Northcutt, 1987).

The dopaminergic/catecholaminergic innervation of the striatum (basal ganglia) and nucleus accumbens (limbic system) by A9–A10 neurons in Bombina orientalis arises primarily from distinct retromammillary, posterior tubercular, and mesencephalic cell groups, which is in accordance with previous studies about the anuran brain (Marín, González, & Smeets, 1997a; Marín, González, & Smeets, 1997c; Marín, Smeets, & González, 1997b; Marín, Smeets, & González, 1997d; Marín, Smeets, & González, 1998). In anamniotes the A9 and A10 cell groups seem to exhibit a higher degree of topographic separation (reviewed by Smeets & González, 2000; Smeets et al., 2000). Although, a differentiation into a nigrostriatal pathway by substantia nigra dopaminergic A9 neurons and a mesolimbic and mesocortical pathway by A10 neurons of the VTA in mammals was long recognized as an oversimplification (reviewed by Björklund & Dunnett, 2007), areas exhibiting properties corresponding to those found in mammals can also be observed in anuran amphibians (present study). Neurons in the A9–A10 cell groups of anamniotes were previously described as intermixed and lacking the laterally migrated substantia nigra (Smeets et al., 2000). The present study does not contradict this assessment, but rather describes additional properties of the anuran dopaminergic system congruent to those found in mammals.

Our approach emphasizes the cytoarchitectonic features of the dopaminergic system, the interconnectivity with the habenular nuclei, as well as forebrain and spinal cord projections (Figure 8). Based on available data, comparisons will be made to lampreys, chondrichthyes, teleosts, reptiles, birds, and mammals:

1. The projections of the LHb to the SNc and VTA in mammals (Akagi & Powell, 1968; Araki et al., 1988; Herkenham & Nauta, 1979; Way & Kaelber, 1969) and onto dopaminergic VTA neurons (Brinschwitz et al., 2010; Omelchenko et al., 2009), resembles the projections of the VHb to catecholaminergic TPdm and TPdl neurons in Bombina orientalis (Figure 7a). This appears to be a phylogenetically old connection, because labeled fibers from the LHb homolog in lampreys can also be found close to catecholaminergic neurons in the nucleus of the posterior tubercle (Stephenson-Jones et al., 2012). This probably also applies to reptiles, because following LHb lesion sparse terminal degeneration can be found in the ventrolateral tegmentum, regarded as the substantia nigra homolog of mammals (Distel & Ebbesson, 1981). In contrast, no connections of the habenula to the teleostean dopaminergic system could be found in the zebrafish (Amo et al., 2010), goldfish (Villani et al., 1994), and rainbow trout (Yáñez & Anadón, 1996). Because these connections are already present in lampreys, a secondary loss in teleosts could be considered.

2. The afferent projections of the mammalian MHB to the ventral tegmentum (Akagi & Powell, 1968; Cuello, Emson, Paxinos, & Jessell, 1978), or more precisely to the interfascicular nucleus of the ventral...
tegmental area (IF; Phillipson, 1979), corresponds to the projection of the anuran DHb to the mesencephalic IF (Figure 7a). Due to the absence of detailed studies in other tetrapods, this connection can only be hypothesized for reptiles and birds.

(3) The probable differential innervation of the MHB and LHb by the IF and medial VTA, respectively (Phillipson & Griffith, 1980; Phillipson & Pycock, 1982), tends to coincide with differential innervation of the DHb and VHb by the IF and TPdm/TPdl (present study; Freudenmacher et al., 2019). Whereas dopaminergic fibers in the habenula are described in reptiles (Smeets, Hoogland, & Voorn, 1986), the precise origin of these projections is not defined. Data from the literature imply comparable habenula input from the posterior tubercle in teleosts (Turner et al., 2016; Yáñez & Anadón, 1996), sharks (Giuliani, Minelli, Quaglia, & Villani, 2002), and lampreys (Pérez-Fernández et al., 2014), although a differential innervation is not present.

(4) The nucleus accumbens projection of IF and paranigral VTA (Ikemoto, 2007; Phillipson & Griffiths, 1985), matches the nucleus accumbens projection of the IF and TPdl of the present (Figure 8) and previous studies (Smeets et al., 2000). While a separate "nigrostriatal" and "mesolimbic" forebrain projection is not described in nontetrapod anamniotes, comparable data can be found in both reptiles and birds (González, Russchen, & Lohman, 1990; Reiner, 2002).

(5) The majority of dopaminergic cells innervating the caudate-putamen of mammals are located in the ventral tier of the SNc (reviewed by Björklund & Dunnett, 2007), which is represented mostly by the ventral TPdm of anurans (Figure 8). In lamprey, chondrichthyes, and teleosts, this nigrostriatal projection is represented by dopaminergic neurons in the posterior tubercle (Northcutt et al., 1988; Pérez-Fernández et al., 2014; Reiner & Northcutt, 1987; Rink & Wullimann, 2001; Wullimann, 2014).

(6) The direct spinal cord projection of A10 and A11 dopaminergic neurons (including posterior and dorsal hypothalamic areas) in the mouse (Qu et al., 2006; Sharples, Koblinger, Humphreys, & Whelan, 2014) is in agreement with the descending catecholaminergic projections to the spinal cord in amphibians from the ventrolateral posterior tubercle (i.e., Rm in the present study; Sánchez-Camacho, Martin, ten Donkelaar, & González, 2002), as well as the Rm and rostral TPdl but not TPdm of the present study (Figure 8). In the lamprey, catecholaminergic spinal projecting neurons were found in the mamillary region and paratubercular nucleus (Barreiro-Iglesias, Villar-Cerviño, Anadón, & Rodicio, 2008) and in zebrafish in the posterior tubercle and hypothalamus (Tay, Ronneberger, Ryu, Nitschke, & Driever, 2011).

(7) The innervation of the supramamillary nucleus (i.e., retromamillary region according to current literature; Puelles, 2019) by the LHb in rat

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**FIGURE 8** The anuran dopaminergic and GABAergic systems exhibit properties comparable to the mammalian VTA, SNc/SNr and RMTg. (a, b) Overview of the organization of the anuran dopaminergic and GABAergic systems. (c) Comparison between the lamprey, anuran, and mammalian dopaminergic pathways and location of GABAergic cell populations. Lamprey and anuran brain (c) redrawn after Nieuwenhuys et al. (1998). For abbreviations see list [Color figure can be viewed at wileyonlinelibrary.com]
(Herkenham & Nauta, 1979; Kiss, Csáki, Bokor, Kocsis, & Kocsis, 2002) and cat (Araki et al., 1988), should be in compliance with Vhb innervation of the Rm (Figure 7a). Detailed studies are missing in other vertebrates, but these connections could be in part comparable to projections to the hypothalamus in reptiles and lampreys (Distel & Ebbesson, 1981; Stephenson-Jones et al., 2012).

8. The projection of the VTA/SNc to the prefrontal cortex (reviewed by Björklund & Dunnett, 2007), could be comparable to projection of the rostral and intermediate TPd to the rostral pallium (Figure 8). It was previously argued, that the anuran rostral pallium, with its projections to the septum, nucleus accumbens, and anterior dorsal striatum, resembles—in terms of connectivity—the mammalian frontal cortex and that an "executive loop" may exist in anuran amphibians (Roth, Laberge, Mühlenbrock-Lenter, & Grunwald, 2007).

In birds, dopaminergic projection from the VTA/SNc reach the dorsolateral nidiopallium (Güntürkün, 2005; Wynne & Güntürkün, 1995). Therefore, it seems that the "mesocortical" pathway had already evolved when the amphibian line of evolution diverged from that leading up to mammals, reptiles, and birds. However, the highly divergent evolution of the pallium makes a 1:1 comparison between specific pallial substructures in different species difficult. It is therefore possible, that this dopaminergic projection has been adapted differently in amphibians, mammals, and sauropsids (i.e., reptiles and birds).

9. The catecholaminergic innervation of the septum by the VTA in rats (Lindvall & Stenevi, 1978; Moore, 1978; Osteniente, Geffard, & Calas, 1984) resembles the septum innervation mainly by catecholaminergic TPdm and mesencephalic neurons in anurans (Rodén, Endepols, & Walkowiak, 2005; Sánchez-Camacho, Peña, & González, 2003). In birds, the lateral septum receives most of its dopaminergic input from the VTA, while the medial septum is innervated by axons from the SNc (Kitt & Brauth, 1986).

10. The midline IF of the rat is localized dorsal to the interpeduncular nucleus (IP) and its dopaminergic neurons density may be characterized as the greatest in the ventral midbrain (Ikemoto, 2007), whereas the anuran midline IF is located rostral to the IP and shows similar cytoarchitectonic characteristics.

11. Histaminergic neurons in mammals can only be found in the tuberomamillary nucleus (Schwartz, Arrang, Garbarg, Pollard, & Ruat, 1991; Wada, Inagaki, Yamatodani, & Watanabe, 1991), which could be comparable with the ventral part of the mammillary area of anurans (Figure 7b). A connection of the habenula with this area—as demonstrated in the present study—is not described in mammals but is comparable to a hypothalamic connection in lampreys (Stephenson-Jones et al., 2012).

12. Serotonergic neurons in reptiles can be found in the organon periventriculare hypothalamicum (Smeets & Steinbusch, 1988), or in the NPv of anuran amphibians (present study), while serotonin-accumulating nerve cells can be found in the rat hypothalamus (Kent & Sladek Jr., 1978). In the lamprey (Stephenson-Jones et al., 2012), habenula connections to serotonergic neurons in the ventral mammillary area (vMAM) are described, possible comparable to the VHb innervation of the NPv in the present study.

A gradual demarcation between VTA and SNc homolog structures, the mammillary bodies, and hypothalamus is therefore possible in anuran amphibians. In summary, the TPdl is comparable to the paranigral VTA (VTApn), the ventral TPdm to the ventral tier of the SNc, the midbrain dopaminergic cell group to the IF, the ventral mammillary area to the tuberomamillary nucleus, whereas the retromamillary area also includes the superficial layer of the mammillary area of previous studies (Figure 8). All these features are in favor of the phylogenetic conservation of the MHb/LHb-VTA/SNc circuitry.

4.3 | The anuran RMTg

In our present experiments, we have focused on the exact topographic determination of the anuran RMTg by further studying its previously identified anatomical features. The anuran superficial mammillary “RMTg” is prominently GABAergic in origin and projects to the anuran VTA/SNc homologs (IF excluded), as well as to the serotonergic raphe nuclei (Figure 7c), which resembles the situation in mammals. The rodent RMTg is additionally characterized by dense opioid receptor and somatostatin immunoreactivity, as well as by GAD67 positive neurons (Jhou et al., 2009). Some of these characteristics could also be demonstrated in anuran amphibians. In Xenopus laevis, the proposed RMTg homolog is xGAD67 positive (Brox, Puelles, Ferreiro, & Medina, 2003). Additionally, the dorsal infundibular hypothalamus in general is immunopositive for somatostatin (Laquerrière et al., 1989; Vallarino, Mathieu, D’Aniello, & Rastogi, 1998). Further input to the proposed anuran RMTg arises in the VHb (Figure 7c), which is—as its mammalian counterpart (Gonzáles et al., 2012)—topographically organized. This input was by previous authors interpreted as a habenulo-hypothalamic projection (Domínguez et al., 2014; Neary, 1995), but can also be explained by the labeling of the hbsm. A previous study in Xenopus laevis investigated the connections of the tuberal and mammillary regions (Domínguez et al., 2014). Most of the described hodological properties also apply to the mammalian RMTg, including connections with the nucleus accumbens, amygdala, preoptic area, septum, bed nucleus of stria terminalis, hypothalamus, and mesencephalic tegmentum (Jhou et al., 2009). The apparent differences in topographic location of these structures could of course raise questions about the homology, but an emphasis on the phylogenetic development could account for the topographic relationships. In lampreys, the LHB homolog projects indirectly via the GABAergic dorsal mammillary area (dMAM) to dopaminergic and serotonergic neurons (Stephenson-Jones et al., 2012). Furthermore, the dopaminergic neurons projecting to the striatum in lampreys are located in the diencephalon, dorsal to the dMAM.

4.4 | A hypothesis about the evolution of the tetrapod dopaminergic system

With the parallel expansion of the pallium/cortex and limbic system in tetrapods, the afferent and efferent connectivity of the dopaminergic system increased. Whereas in lampreys "nigrostriatal" and spinal cord
projections are present, the tetrapod system also includes a "mesolimbic" and "mesocortical" pathway. With the appearance of the neocortex in mammals, the basal ganglia output to the motor cortex via the thalamus gained in significance (Groenewegen, Voorn, Berendse, Mulder, & Cools, 2009). This is correlated with a topographic relocation of dopaminergic and GABAergic cell populations, as illustrated in Figure 8c showing the origin of dopaminergic pathways and the position of the GABAergic RMTg and substantia nigra pars reticulata (SNr). Anuran amphibians therefore represent the transition of a diencephalic RMTg-VTA/SNc circuitry in anamniotes to a mesencephalic system in amniote vertebrates. Dopaminergic domains exhibiting properties corresponding to the mammalian parapinalg VTA, ventral tier of the SNc, interfascicular nucleus of the VTA, and supramammillary/retromamillary region are already present in anuran amphibians. Thus, most features of the dopaminergic system were already evolved when the amphibian line of evolution diverged from that leading up to amniote tetrapods.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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