The habenula as an evolutionary conserved link between basal ganglia, limbic, and sensory systems—A phylogenetic comparison based on anuran amphibians

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Abstract

Based on anatomical and functional data, the habenula—a phylogenetically old brain structure present in all vertebrates—takes part in the integration of limbic, sensory, and basal ganglia information to guide effective response strategies appropriate to environmental conditions. In the present study, we investigated the connections of the habenular nuclei of the oriental fire-bellied toad, Bombina orientalis, and compared them with published data from lampreys, chondrichthyes, teleosts, reptiles, birds, and mammals. During phylogenetic development, the primordial habenula circuitry underwent various evolutionary adaptations and in the tetrapod line, the circuit complexity increased. The habenula circuitry of anuran amphibians, descendents of the first land-living tetrapods, seem to exhibit a mix of ancient as well as modern features. The anuran medial and lateral habenula homologs receive differential input from the septum, nucleus of the diagonal band of Broca, preoptic area, hypothalamus, rostral pallium, nucleus accumbens, ventral pallidum, and bed nucleus of the anterior thalamus.

Abbreviations: 3V, third ventricle; A, anterior thalamic nucleus; Ac, anterior commissure; Acc, nucleus accumbens; Ad, anterodorsal thalgmental nucleus; adhyp, adenohypophysis; Av, anteroven tral thalgmental nucleus; BN, bed nucleus of the pallial commissure; BON, nucleus of the basal optic root; BST, bed nucleus of the stria terminalis; C, caudal; C, central thalamic nucleus; Ch, cerebellum; CeA, central amygdala; ch, habenular commissure; cpal, pallial commissure; cb, lateral corticothalmic tract; d, dorsal; DH, dorsal hypothalamus; DHB, dorsal habenula; DL, dorsolateral prethalamus; dMAM, dorsal mamilary region; DP, dorsal pallidum; Ea, anterior entopeduncular nucleus; EP, entopeduncular nucleus; ep, episphysis; Ep, posterior entopeduncular nucleus; fpl, external plexiform layer of the main olfactory bulbs; fr, fasciculus retroflexus; frf, caudal fascicular retroflexus; frf, rostral fascicus retroflexus; Hb, habenula; Hif, habenulo-interfascicular tract; hipp, habenulo-interpeduncular tract; hipp, rostral habenulo-interpeduncular tract; hrb, habenulo-raphe tract; hsm, habenulo-superficial-mamillary tract; hstp, habenulo-posterior-tuberclate tract; If, interpeduncular nucleus of the ventral teggmental area; Igl, internal granule layer of the main olfactory bulbs; III, nucleus of the oculomotor nerve; IP, interpeduncular nucleus; Ism, isthmic nucleus; L, lateral thalamic nucleus; LDIHb, left dorsolateral habenula; LDmHb, left dorsomedial habenula; LVHb, left ventral habenula; LA, lateral amygdala; LC, locus coeruleus; LDT, laterodorsal telligental nucleus; lb, lateral forebrain bundle; LH, lateral hypothalamus; LHB, lateral habenula; Lp, lateral pallium; ls, lateral septum; Mam, mammillary area; MeA, medial amygdala; mFB, medial forebrain bundle; MFB, medial habenula; M, medial prethalamus; ml, molecular layer of the main olfactory bulbs; MOb, main olfactory bulbs; Mp, medial pallium; Ms, medial septum; Mv, ventral part of the medial prethalamus; Ndb, nucleus of the diagonal band of Broca; NME, nucleus of the medial longitudinal fasciculus; Npc, nucleus of the posterior commissure; NPv, nucleus of the periventricular organ; OB, olfactory bulbs; olv, superior olivary; optl, lateral optic tract; opm, medial optic tract; OT, optic tectum; otA, anterior optic tract; otR, lateral optic tract; otHM, medial optic tract; P, posterior thalamic nucleus; PAG, periaqueductal gray; PBA, parabrachial nucleus; Pd, postertodorsal telligental nucleus; PFC, prefrontal cortex; Pcn, pineal organ; prerv, pars nervosa (pituitary gland); PoA, preoptic area; PTE, prethalamic eminence; Pv, postertoventional telligental nucleus; R, rostral; rDHB, right dorsal habenula; rVHb, right ventral habenula; RaC, superior central raphe nucleus; RaD, dorsal raphe nucleus; RaM, nucleus raphe magnus; Rp, nucleus raphe pontis; Rm, retromamillary area; RMTg, rostromedial telligental nucleus; Rp, rostral pallium; Sc, central septal nucleus; Sc, superior colliculus; SC, suprachiasmatic nucleus; Sdfc, dorsocaudal suprachiasmatic nucleus; Sl, dorsal septal nucleus; sfgs, stratum fibrasum et griseum superficiale; sgc, stratum griseum centrale; SGP, stratum griseum periventriculare; Sl, dorsolateral septal nucleus; Sm, striatolateral septal nucleus; SNC, substantia nigra pars compacta; SRn, substantia nigra pars reticulata; ST, striatum; STR, striatopallidal system; Sum, supramamillary region; Th, nucleus laminaris of the torus semicircularis; TM, mesencephalic tectum; Tm, nucleus magnocellularis of the torus semicircularis; Tp, nucleus principalis of the torus semicircularis; TP, posterior tubercle; TPd, dorsolateral nucleus of the posterior tubercle; TPdM, dorsomedial nucleus of the posterior tubercle; Tt, torus semicircularis; V, ventral; VHb, ventral habenula; VB, ventral thalgmental prethalamus; Vl, ventral part of the ventral lateral prethalamus; vMAM, ventral mamilary region; VP, ventral pallium; VLG, ventral lateral geniculate; VTA, ventral telligental area; Zip, periventricular nucleus of the zona incerta.

Arndt von Twickel and Wolfgang Walkowiak contributed equally to this study.

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of the stria terminalis. Additional input arises from a border region in the ventral pre-thalamus, here discussed as a putative homolog of the entopeduncular nucleus of rodents. The habenular subnuclei also differentially innervate the interpeduncular nucleus, raphe nuclei, substantia nigra pars compacta and ventral tegmental area homologs, superficial mamillary area, laterodorsal tegmental nucleus, locus coeruleus, inferior and superior colliculus homologs, hypothalamus, preoptic area, septum, nucleus of the diagonal band of Broca, and main olfactory bulb. It seems likely that the main connectivity between the habenula and the basal ganglia, limbic, and sensory systems was already present in the common tetrapod ancestor.

**KEYWORDS**

anuran amphibians, basal ganglia, habenula, limbic system, phylogenetic comparison, RRID: AB_2337244, RRID:AB_2337249, RRID:AB_2572212, sensory system

### 1 | INTRODUCTION

In all vertebrates, the central nervous system has to control basic motivated behaviors that are necessary for survival and reproduction. Anuran amphibians can be of particular interest for studies of nervous systems, because their origins date back to the first land living tetrapods, 245 million years ago (Cannatella, 2006). Their brains can be assumed to resemble those of the amphibian–reptile–like ancestors of present mammals, reptiles, and birds. The habenula, a phylogenetically old brain structure present in all vertebrates, takes part in the regulation of dopaminergic and serotonergic transmission toward forebrain structures via a direct glutamatergic and indirect GABAergic pathway. This feature seems to be conserved from lampreys (Stephenson-Jones, Floros, Robertson, & Grillner, 2012) to mammals (cf. Brown & Shepard, 2016). In many mammals, habenula lesions result in behavioral changes in relation to pain, stress, anxiety, sleep, reward, and maternal behaviors, as well to cognitive and motor dysfunctions [reviewed by Lecourtier and Kelly (2007) and Hikosaka (2010)]. In mammals, the basic structure of the habenula is similar in terms of location and organization. Lying at the roof of the third ventricle, the habenula is divided into medial (MHB) and lateral parts [LHb; reviewed by Hikosaka (2010)]. These correspond to the dorsal (DHb) and ventral habenula (VHb) of anamniotes, respectively (Guglielmotti & Cristiano, 2006). Structural asymmetries in the MHB/DHb are common among many vertebrate species, but size asymmetries are considerably smaller in mammals (Wree, Zilles, & Schleicher, 1981). However, the extent of the phylogenetic conservation of the habenula circuitry among vertebrates is strongly debated (cf. Amo et al., 2010; Kemali, Guglielmotti, & Gioffre, 1980; Laberge & Smith, 2017; Stephenson-Jones, Floros, et al., 2012). The primary function of the habenula is hypothesized to suppress motor activity under adverse conditions (Hikosaka, 2010). In the rat, mouse, and macaque, stimulation of the LHb inhibits dopaminergic neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA; Christoph, Leonzio, & Wilcox, 1986; Matsumoto & Hikosaka, 2007; Lammel et al., 2012; Stamatakis & Stuber, 2012) and in rats also serotonergic neurons in the raphe nuclei (Wang & Aghajanian, 1977), which appears to be mediated by GABAergic neurons in the rostromedial tegmental nucleus (RMTg; Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009; Hong, Jhou, Smith, Saleem, & Hikosaka, 2011). Counterparts of the VTA and SNc have been described in anuran amphibians, including dopaminergic neurons in the ventral tegmentum and posterior tubercle (Marín, González, & Smeets, 1997a, 1997b; Mariño, Smeets, & González, 1997a). Recent studies in mammals focused on the LHb-RMTg-VTA/SNc/raphe circuitry and its involvement in reinforcement learning and control of punishment-avoidance behaviors by regulation of serotonergic and dopaminergic signals toward hippocampus, frontal cortex, and nucleus accumbens [reviewed by Lecourtier and Kelly (2007) and Hikosaka (2010)]. However, direct connections to sensory, basal ganglia, limbic, and brainstem regions imply a possible further operating principle besides signaling aversive situations (cf. Herkenham & Nauta, 1979; Hikosaka, 2010). The habenula is therefore an ideal candidate structure in exerting control over the flow of information in key brain areas involved in the sensory processing, the evaluation of behavior, the selection of motor programs, and the integration of the internal physiological state. In comparison, the habenula circuitry of lampreys seems to be organized less complex than in mammals (cf. Herkenham & Nauta, 1979; Stephenson-Jones, Floros, et al., 2012). Therefore, the question arises if the anuran amphibian habenula circuitry reflects—if any—the situation found in lampreys or mammals. In the present study, an emphasis was placed on the VHb, because its connectivity is supposedly the least preserved among vertebrates (cf. Amo et al., 2010; Laberge & Smith, 2017). Consequently, here, the hypothesis was tested that the habenula of amphibia has network structures that can be regarded to reflect the ancestral state of the tetrapod habenula. The present investigation aims to compare the structural properties of the anuran habenula with the habenula of extant lampreys, chondrichthyes, teleosts, reptiles, birds, and mammals at the level of the connectivity to provide insights into habenula evolution and function.
2 | MATERIALS AND METHODS

2.1 | Isolated brain preparations

Subject of investigation was the isolated brain preparation of the Oriental fire-bellied toad, Bombina orientalis (Luksch, Walkowiak, Muñoz, & ten Donkelaar, 1996). Bombinatoridae are of interest because they belong to a basal group in anuran phylogeny (Frost et al., 2006; Pyron & Wiens, 2011). The project is registered after §4 of the German Animal Protection Act under the title "NeuroAnura," approval number 4.16.004. Sixty-four 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) tricaine methanesulfonate (MS-222; Sigma-Aldrich, Cat# E10521) in tap water for 10 min (Luksch et al., 1996; Ohr, 1976). The body temperature was cooled down on ice, the medulla severed with a surgical chisel and the head removed. Brains were isolated by a ventral approach, transferred into a dish with Ringer’s solution (75 mM NaCl, 25 mM NaHCO₃, 2 mM CaCl₂, 2 mM KCl, 0.5 mM MgCl₂, and 11 mM D-glucose), which was adjusted with carbogen (95% O₂, 5% CO₂) to a pH of 7.4 and stored at 5 ºC.

2.2 | Anatomical tracings

For the anatomical reconstruction of the anuran habenula circuitry, small deposits of neuronal tracers were placed in different subnuclei of the habenular complex. Complementary application of tracer was performed to confirm afferent and efferent structures. The neuronal tracer Neurobiotin (Vector Laboratories, Cat# SP-1120), a Dextran tetramethylrhodamine-isothiocyanate-dextran (Sigma Aldrich, Cat# T1037) (20% [wt/vol] in distilled water) were pressure injected into various brain areas in whole brain or half-brain preparations (transected mid-sagitally) using glass micropipettes. The sites of tracer applications reconstructed in the histological sections (see below) are shown in Table 1. Following applications, the brains were kept 24–48 hr at 5 ºC in Ringer’s solution to allow the anterograde and retrograde transport of the tracers. The Ringer solution was changed at regular intervals to ensure a stable pH and oxygenation.

2.3 | Histochemical procedures

The brains were fixed overnight at 5 ºC by submersion in 4% (vol/vol) paraformaldehyde and 14% (vol/vol) saturated picric acid in 0.1 M phosphate buffer (PB). Because the brains were also used for immunohistochemical studies (Freudenmacher, Schauer, Walkowiak, & von Twickel, 2019), glutaraldehyde was added or 1-ethyl-3-(3-diimethylamino-propyl)-carbodiimide hydrochloride (Sigma-Aldrich, Cat# E7750) was used to fix the brains. The brains then were cryoprotected with 20% (wt/vol) sucrose in PB for 3–12 hr, embedded in Tissue-Tek mounting medium (Sakura, Cat# 4583) and rapidly frozen. Transverse sections (20–30 μm) were made with a cryostat (Leica CM 3050 S). The sections were collected on adhesion slides (Superfrost Plus, Thermo Scientific) and stored at −20 ºC until further processing. Before further treatment, the sections were dried for 45 min at 37 ºC. The slices were rinsed with phosphate-buffered saline (PBS) and incubated with 1:500 deep-red fluorescent Nissl stain (Molecular Probes, Cat# N21483, RRID:AB_2337244) in 1% (wt/vol) BSA (0.3% [vol/vol] Triton-X 100 in 0.1 M PB). The slices were washed with PBS and covered with glycerol containing 2.5% (vol/vol) diazabicyclooctane and a coverslip.

2.4 | Analysis of histological stainings

The sections were examined with a fluorescence microscope (coupled to a digital camera, Zeiss AxioCam HR) or a confocal...
3.1 | Connectivity of the habenula

Based on direct and complementary injections, the afferent and efferent connectivity of the habenula (Hb) was identified. The anuran habenula is asymmetrical, with an enlarged left dorsal habenula, and is composed of five distinct nuclei: the left dorsolateral (l.DlHb), left dorsomedial (l.DmHb), left ventral (l.VHb), right dorsal (r.DHb), and right ventral habenula (r.VHb; Figure 1). Seventeen animals received unilateral injections into the different subnuclei. Data of the connectivity of 10 representative experiments are summarized in Tables 2 and 3 and form the basis of Figure 2. The following result section is divided into two parts and describes first afferent and then efferent structures. References are provided to put the results into the context of current anatomical classifications.

3.2 | Afferents of the habenular nuclei

Application of Neurobiotin into the habenula (Figure 2: 7) resulted in labeling of various fiber tracts including the lateral corticohabenular (chtl) lateral (otl), and anterior olfactory (habenular) tracts (ota) of Herrick (1927) which converge from rostral and lateral positions (Figure 2: 6, 7) in the stria medullaris (sm) to form a thick fiber bundle. Additional fibers contributing to the stria medullaris were found in the medial olfactory (habenular) tract (otm), which first can be distinguished in transverse sections at the level of the medial amygdala (MeA; Figure 2: 5). At a more rostral point, the lateral corticohabenular tract merges with the lateral olfactory tract (Figure 2: 3, 4) while the medial olfactory tract joins the medial forebrain bundle (mbf). The anterior olfactory tract remains in a ventral position. More dorsally, the medial corticohabenular tract (chtm) is found. The cthl can be distinguished from the otl again at the position of the rostral pallium (rP). Furthermore, in most cases the lateral forebrain bundle (lfb) was labeled (Figure 2: 3, 4). Afferent and possibly efferent fibers caudal to the Hb also included the lateral (optl) and medial (optm) optic tracts. The latter takes a dorsal course from the optic tectum (OT; superior colliculus homolog) laterally along the epiphysis (ep) to the habenula (Figure 2: 15–8). Part of the optl is likely to fuse with the fasciculus retroflexus (fr) at a diencephalic level (Figure 2: 9) and branches at the level of the OT (Figure 2: 15) into single axons. Afferent labeled fibers also originate from different subnuclei of the torus semicircularis (Ts), the anuran inferior colliculus homolog (pars laminaris: Tl; pars magnocellularis: Tm; pars principalis: Tp; Figure 2: 14–15).

3.3 | Input from bed nucleus of the stria terminalis and ventral pallidum

Most of the backfilled perikarya were located ipsilateral to the injection side but some were also found in corresponding contralateral areas.
### TABLE 2  Retrograde cell labeling after tracer injection into the habenula

| Case | Injection site | MOB | rP | Acc | Ls | Ndb | Str | Str/DP | DP | LA | MeA | CeA | VP/BST | BST | Mp | Poa | BN | PTE |
|------|----------------|-----|----|-----|----|-----|-----|--------|----|----|-----|-----|--------|-----|----|-----|----|-----|
| Hb1  | r.DHb, r.VHb, sm, ch | ++++ | ++ | ++ | +++ (c) | + | + | ++ | ++++ | + | ++ | ++++ | + | ++++ | ++ | ++++ | ++ | ++++ |
| Hb2  | Half brain, r.DHb, r.VHb | - | - | - | -/+ (r) | - | - | + | - | - | - | - | - | - | - | - | - |
| Hb3  | l.DHb, l.VHb, sm | - | - | ++ (c) | -/+ | -/+ | - | + | -/+ | - | -/+ | + | -/+ | + | + | ++++ | + |
| VhB1 | Caudal l.VHb, sm | + | -/+ | -/+ | ++ (c) | -/+ | - | - | ++ | + | + | ++++ | + | ++++ | ++ | ++++ |
| VhB2 | Half brain, l.VHb, sm | + | -/+ | + | - | - | - | + | + | + | -/+ | + | + | ++++ | + |
| VhB3 | Half brain, r.VHb | - | -/+ | + | -/+ (c) | - | + | + | - | - | - | + | + | + | -/+ | ++++ |
| VhB4 | Half brain, l.DHb | + | -/+ | - | + | + | -/+ | + | -/+ | + | + | ++++ | + | + | + | + | + |
| DmB1 | l.DmHb, ch | + | - | + (r) | - | - | -/+ | - | - | -/+ | - | - | - | - | + | -/+ |
| DmB2 | Half brain, l.DHb | ++ | - | - | -/+ (c) | - | - | - | ++ | -/+ | -/+ | -/+ | - | - | + | + | ++++ |
| DmB3 | Half brain, caudal r.DHb | - | - | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | - | -/+ | - | nd | - | - | - | + |
| Sm  | Stria medullaris, caudal | ++ | - | + (c) | - | - | -/+ | - | - | -/+ | - | - | - | - | ++++ | + | + |

| Case | Injection site | Mv | VLv | VLG | Ep | Sc | A | L | C | Npc | DH | VH | TPdm/dl | Ts | OT | IF | IP | RaM |
|------|----------------|----|-----|-----|----|----|---|---|---|----|----|----|--------|----|----|----|----|-----|
| Hb1  | r.DHb, r.VHb, sm, ch | ++++ | +++ | +++ | ++ | -/+ | +++ | + | ++ | + | -/+ | + | -/+ | -/+ | -/+ | -/+ | -/+ |
| Hb2  | Half brain, r.DHb, r.VHb | +++ | +++ | +++ | - | + | - | - | - | -/+ | - | - | -/+/+ | - | - | - | - | n.d |
| Hb3  | l.DHb, l.VHb, sm | +++ | +++ | +++ | ++ | + | + | - | -/+ | + | -/+ | -/+- | -/+/+ | -/+- | -/+- | n.d |
| VhB1 | Caudal l.VHb, sm | +++ | +++ | +++ | ++ | + | +++ | + | + | + | -/+ | -/+ | -/+ | -/+ | -/+ | - | - |
| VhB2 | Half brain, l.VHb, sm | +++ | +++ | +++ | ++ | + | + | - | - | -/+ | + | + | + | -/+ | -/+ | - | - |
| VhB3 | Half brain, r.VHb | +++ | +++ | +++ | ++ | -/+ | + | - | - | -/+ | + | -/+ | -/+ | -/+ | -/+ | -/+- | -/+- |
| VhB4 | Half brain, l.VHb | +++ | +++ | +++ | ++ | + | + | - | - | -/+ | + | -/+ | -/+- | -/+- | -/+- | -/+- | -/+- |
| DmB1 | l.DmHb, ch | + | ++ | ++ | + | - | - | - | - | -/+ | + | -/+ | -/+- | -/+- | -/+- | -/+- | -/+- |
| DmB2 | Half brain, l.DHb | ++ | +++ | +++ | ++ | + | + | - | - | -/+ | + | -/+ | -/+- | -/+- | -/+- | -/+- | -/+- | n.d |
| DmB3 | Half brain, caudal r.DHb | - | + | + | - | -/+ | -/+ | - | - | -/+ | + | -/+ | -/+- | -/+- | -/+- | -/+- | -/+- | n.d |
| Sm  | Stria medullaris, caudal | ++ | -/+ | +++ (c) | - | - | - | - | - | -/+ | + | + | +++ | + | +++ | + | +++ | n.d |

**Note:** -, no cells; -/+ , occasional cell; +, less than 5; ++, less than 10; ++++, less than 15; ++++, less than 30; +++++, less than 50; ++++, less than 100; ++++++, more than 100 cells per section; n.d., no data. Abbreviations: A, anterior thalamic nucleus; Acc, nucleus accumbens; BN, bed nucleus of the pallial commissure; C, central thalamic nucleus; CeA, central amygdala; ch, habenular commissure; DH, dorsal hypothalamus; Ep, posterior entopeduncular nucleus; IF, interfascicular nucleus of the ventral tegmental area; IP, interpeduncular nucleus; L, lateral thalamic nucleus; l.DHb, left dorsal habenula; l.DlHb, left dorsolateral habenula; l.DmHb, left dorsomedial habenula; l.VHb, left ventral habenula; LA, lateral amygdala; Ls, lateral septum; MeA, medial amygdala; MOB, main olfactory bulb; Mp, medial pallium; Mv, ventral part of the medial prethalamus; Ndb, nucleus of the diagonal band of Broca; Npc, nucleus of the posterior commissure; OT, optic tectum; Poa, preoptic area; PTE, prethalamic eminence; r.DHb, right dorsal habenula; r.VHb, right ventral habenula; RaM, nucleus raphe magnus; rP, rostral pallium; Sc, suprachiasmatic nucleus; sm, stria medullaris; Str, striatum; Str/Dp, striatopallidal system; TPdm/dl, dorsomedial and dorsolateral posterior tubercle; Ts, torus semicircularis; VH, ventral hypothalamus; VLG, ventrolateral geniculate; VLv, ventral part of the ventrolateral prethalamus; VP/BST, ventral pallidum and bed nucleus of the stria terminalis.
Within the telencephalic–diencephalic boundary, injections including the VHB resulted consistently in retrogradely labeled cells in bed nucleus of the stria terminalis (BST). In some cases, moderate to abundant cell labeling was found in the medial (MeA), lateral (LA), and more rostral in the central (CeA) amygdala projecting via the amygdalo-habenular tract of Herrick (Table 1; Figure 2: 3, 4). These connections were characterized as BST/VP in this study (Figure 2: 4; Figure 3e; Table 2).

### 3.4 Input from preoptic area and suprachiasmatic nucleus

Labeled cells at the level of the MeA (Figure 1: 5) were found in the preoptic area (Poa), some of them on the contralateral side (Figure 3g). Their axons course through the preoptic tract and anterior commissure (ac). Few cells in the suprachiasmatic nucleus (Sc) were found at the level of the optic chiasm (Figure 2: 7, 8; Figure 3j; Table 2), the axons take a medial course.

### 3.5 Input from septum and nucleus of the diagonal band of Broca

Some of the labeled mfb fibers can be attributed to retrogradely labeled neurons located in the lateral septum (Ls) and nucleus of the diagonal band of Broca (Ndb; Figure 2: 3, 4; Figure 3c,e). Marked cells appear in a fairly random distribution and were scattered over the whole septum. Table 2 therefore summarizes the different septal nuclei (dorsal: Sd; dorsolateral: Sdl; ventrolateral: Slv) in its rostral (r) or caudal (c) proportions. Most labeled cells were located in the caudal Ndb. An obviously different innervation of the dorsal (DHb) or ventral (VHb) habenula by the Ls or Ndb could not be determined (Table 2).

### 3.6 Input from nucleus accumbens and rostral pallium

Injections located in the whole or ventral habenula showed labeled neurons in the nucleus accumbens in a distributed pattern (Acc;
FIGURE 2  Schematic view of anterogradely and retrogradely labeled fibers (red) and labeled neurons (blue) in the brain of *Bombina orientalis* after unilateral application of Neurobiotin to the habenula. The figures show transverse sections through the brain at levels indicated in the inset (lower right corner). The cytoarchitecture is indicated in gray. For abbreviations, see list.
FIGURE 3  Confocal images of anterogradely and retrogradely labeled structures ipsilateral or medial to the tracer injection into the habenula. Injection in the habenula resulted in labeled cells in (a) rostral pallium; (b) nucleus accumbens; (b,c,e) lateral septal nuclei; (d) striatopallidal system; (d,f) amygdala and nucleus of the diagonal band of Broca; (f) dorsal pallidum; (g) preoptic area; (h,i) bed nucleus of the pallial commissure and prethalamic eminence with labeled axons entering the stria medullaris; (j,k) the Mv/ VLv/VLG layer with retrogradely labeled fibers which loop around the lateral forebrain bundle; (j) suprachiasmatic nucleus; (l,m) thalamus; (n) alar hypothalamus; (p,q) dorsal tuberal hypothalamus; (q) posterior tubercle; (r) nucleus of the posterior comissure; (s) interfascicular nucleus; (t) optic tectum; (u) torus semicircularis; and (x) interpeduncular nucleus. The lateral contingent of the fasciculus retroflexus (l,m,p) densely aggregates above the dorsal hypothalamus (p) and proceeds in the habenulo-superficial-mamillar tract to the Sm. Few VHb fibers diverge from the fasciculus retroflexus and reach the central thalamic nucleus (o), preoptic area (g), septum, and nucleus of the diagonal band of Broca (c,e). Densely labeled VHb fibers reach the dorsolateral and dorsomedial posterior tubercle (habenulo-posterior-tubercle tract; p,q). Sparse DHb fibers reach via the habenulo-interfascicular tract the interfascicular nucleus (s). VHb fibers also reach the optic tectum (t) and torus semicircularis (u). Scattered VHb fibers (v) can be observed in the laterodorsal tegmental nucleus and locus coeruleus. Dense fiber labeling in the caudal interpeduncular nucleus (w,x) can only be observed after injection including the left dorsolateral habenula or caudal contingent of the left fasciculus retroflexus (w). Injections limited to the DHb do not label the habenulo-raphe tract (w). Dense VHb fibers reach via the habenulo-raphe tract the RaCS-RaD (x) and RaM (y). Numbers refer to Figure 1. Scale bars 25 μm
Figure 2: 3; Figure 3b; Table 2). Their axons follow the medial forebrain bundle. Complementary tracer applications into the Acc (n = 3) revealed anterograde labeled fibers terminating in the VHb. Fibers terminating in the DHb can be explained by fibers bypassing the Acc, originating from the Ls (Figure 4a). Occasional, but rather sparse, marked cells were also found ipsilateral in the rostral pallium (rP) after applications including the VHb (Figure 2: 2; Figure 3a; Table 2). Labeled axons follow the lateral corticohabenular tract. Tracer injections into the rP (n = 3) revealed few anterogradely labeled fibers in the VHb (Figure 4b). Unlike Neurobiotin application into the olfactory bulbs (Figure 4c), injections restricted to the rP did not stain fibers crossing the habenula commissure. Scale bars 25 μm.

3.7 | Labeled cells in the olfactory bulbs
Most tracer injections that label the habenula and a greater proportion of the sm, the ch or rather the sm alone, lead to retrogradely marked cells in the internal granule (igl), molecular (ml) and external plexiform (epi) layer (Figure 2: 1, 2) of the main olfactory bulbs (MOB). More limited injections (in most cases by a medial half brain approach) did not result in any labeled neurons (Table 2). As revealed by anterograde tracing experiments (Figure 4c; n = 3), the olfactory bulbs and their associated tracts (Figure 2: 1–7) do not innervate the habenula and cross via the habenula commissure (ch) to the contralateral hemisphere. Furthermore, in two cases, retrogradely labeled neurons in the habenula could be found.

![Image](image_url)

**FIGURE 4** Fiber projections to the anuran habenula. Tracer injection into (a) nucleus accumbens, (b) rostral pallium, (c) main olfactory bulbs, (d) medial pallium, (e) striatum, and (f) optic tecta lead to (a) fibers innervating the ventral habenula, (b) anterogradely labeled axons in the ventral habenula, (c) fibers crossing via the habenula commissure, (d) fibers entering the habenula, but extending toward the habenula commissure, (e) fibers crossing via the habenula commissure and reaching the anterior thalamic nucleus, and (f) fibers innervating the inner habenula neuropil. Scale bars 25 μm.

3.8 | Labeled cells in the medial pallium
Habenula tracer injections, that led to retrogradely labeled cells in the MOB, also often led to labeled neurons in the medial pallium (Mp). Most connections between the medial pallium and the contralateral telencephalon cross over the pallial commissure. However, tracer application into the caudal Mp also showed a small proportion of labeled fibers crossing via the habenula commissure (Figure 4d; n = 4 in N = 2). Some fibers enter the rostral VHb but extend toward the habenula commissure. It seems therefore, that after habenula injections, Neurobiotin was taken up by fibers of passage, which labeled their cells of origin.

3.9 | Labeled cells in the thalamus
The same fibers of passage problem described for labeled neurons in the MOB and Mp following habenula tracer injection applies presumably for labeled cells in the anterior (A), central (C), and lateral (L) thalamic nucleus, which extend their axons laterally along the stria medullaris (Figure 3l, arrowheads). This connection can possibly be attributed to projections to the MeA or to the striatopallidal system (Str/DP).

3.10 | Labeled cells in striatum and striatopallidum
Habenula tracer injections led to labeled cells in the striatum (Str), striatopallidal system (Str/DP), and dorsal pallidum (DP; Figure 2: 4; Figure 3d,f; Table 2). Complementary injections into the striatum (n = 3; Figure 4e) or striatopallidal system (n = 2) showed few fibers entering the habenula, but no axonal varicosities could be observed. Most fibers cross via the habenula commissure and reach the anterior thalamic nucleus. This indicates the labeling of fibers of passage after habenula tracer injection.

3.11 | Input from bed nucleus of the pallial commissure and prethalamic eminence
After habenula tracer injection, labeled neurons can be found in the bed nucleus of the pallial commissure (BN) and the prethalamic eminence (PTE). Retrogradely labeled cells in the BN are densely packed, situated beneath the sm, and have a small, round shape (Figure 2: 5; Figure 3h). Labeled neurons in the PTE are bigger and scattered over a larger area. Their axons extend, as neurons in the BN, dorsally into the sm (Figure 2: 6; Figure 3i).
3.12 | Innervation of the ventral habenula by the ventral prethalamic nucleus

The majority of backfilled cells after injections including the VHb was located in the ventral prethalamic nucleus (Domínguez et al., 2014), here in reference to the old nomenclature by Neary and Northcutt (1983) subdivided into the ventral part of the medial (Mv) and ventral ventrolateral (VLv) prethalamic nucleus (Table 2). Labeled cells in this area form a dense lateral elongated layer with lateral migrated cells in the ventrolateral geniculate (VLG), a round cellular aggregate that is clearly separated. More caudally, the Mv and VLv taper off into the posterior entopeduncular nucleus (Ep), their axons loop around the lateral forebrain bundle and enter the sm at level of the PTE (Figure 2: 6–9; Figure 3i–k). The term entopeduncular nucleus is used in a strictly descriptive sense of lying in the peduncle, not implying homology to the nucleus in mammals. Habenula tracer injections leading to retrogradely labeled neurons in the Mv/VLv layer also lead to marked cells in the Ep nucleus in varying degrees (Figure 2: 9; Table 2). The tracer injection in the caudal sm showed a larger number of labeled neurons in a more caudal part of the Ep, possibly indicating input to the anterior thalamic nucleus.

3.13 | Input from optic tectum and torus semicircularis

Additional but sparse cell labeling upon habenula injection was found in the mesencephalic tectum (tm). Labeled cells were also located in the pretectal nucleus of the posterior commissure (Npc; Figure 3r; Table 2). Retrogradely labeled neurons were found randomly scattered through the gray layers (stratum griseum periventriculare: sgp; centrale: sgc; superficiale: sfgs) of the optic tectum (OT; Figure 2: 14–16; Figure 3t) in both hemispheres. Therefore, Table 2 pools retrogradely labeled cells in the different optic layers. While neurons projecting to the DHb take a mediadorsal course (optdm), axons which take the lateral course (optl) pass into the fasciculus retroflexus (fr) and innervate the inner VhB neuropil (Figure 4f; n = 4 in N = 2). The optic tracts are faintly labeled and because of their rostrocaudal direction best visible in sagittal sections. Marked cells in the midbrain nucleus of the auditory pathway, the torus semicircularis (Ts), are mostly located in the laminar (Tl) and principal nucleus (Tp; Figure 2: 15; Figure 3u). The axons take a mediostral route and assemble with the fr at Level 13 of Figure 2.

3.14 | Input from hypothalamus and posterior tubercle

Hypothalamic input to the habenula arises mostly from infundibular/tuberal levels (Figure 2: 10–14; Figure 3p), but in some cases, input from the alar hypothalamus can also be found (Figure 2: 9; Figure 3n). Neurons in the tuberal dorsal (DH) and ventral hypothalamus (VH) are faintly labeled and the total number (Table 2) possibly underestimated at low magnification. The axons follow the fasciculus retroflexus and diverge at a more rostral region into a transthalamic route. Other cells projecting to the VHb were found in the dorsomedial and dorsolateral nucleus of the posterior tubercle (TPdm/dl; Table 2) and in the retromamillary area (Rm). In this study, the TPDl denotes the superficial layer of the dorsomedial posterior tubercle, whereas the retromamillary area incorporates small portions of the mamillary area of former studies (Domínguez et al., 2014; González & Smeets, 1991; Moreno, Bachy, Retaux, & González, 2004). The used terminology is based on the immunohistochemical and cytoarchitectonic features of the area, as well as the connectivity.

3.15 | Input from interfascicular and interpeduncular nucleus

Injections including the DHb lead in few cases to scattered labeled neurons in the area designated here as the interfascicular nucleus of the ventral tegmental area (IF; Figure 2: 14; Figure 3s; Table 2), formerly described as the mesencephalic basal plate, ventral tegmentum, or ventral tegmental area (Marín, Smeets, & González, 1998a). The term interfascicular nucleus is used in a descriptive sense of lying between the fascicles. Additionally, in some cases, few labeled neurons were found in the interpeduncular nucleus (IP; Figure 2: 17; Figure 3x; Table 2).

3.16 | Input from raphe nuclei

Finally, habenula tracer injections lead in only three cases to retrogradely labeled cells in the nucleus raphe magnus (RaM; Figure 2: 19; Table 2) and in two additional cases to labeled cells lateral or ventral to the RaM, which cannot clearly be assigned to the raphe nuclei. Retrogradely labeled cells explicitly belonging to the superior central and dorsal raphe (RaCS-RaD) complex were not observed.

3.17 | Efferents of the habenular nuclei

Application of Neurobiotin into different parts of the habenula resulted in labeling of different proportions of the fasciculus retroflexus (fr), a compact fiber bundle arising from the habenula. The labeling was most prominent ipsilateral to the application site, but contralateral extending fibers were also observed. In Table 3, the fr is divided into different tracts based on the more rostral or caudal course [fr(r), fr(c)] and the projection targets.

3.18 | The fasciculus retroflexus

Fibers originating from the medial part of the left dorsal habenula (l.DlHb) and the undivided right dorsal habenula (r.DHb) tend to run through a medial diencephalic route (Figure 2: 9; Figure 3m) topographically in ventrocaudal direction (dorsoventral in the alar plate), branching out through the lateral thalamic nucleus (L) into peripheral components. The most lateral proportions of this peripheral route arise from the lateral part of the left dorsal habenula (l.DIHb, data not shown). These fiber tracts [fr(c)] have their origin in the caudal end of the habenula complex, partly coursing through the ventral habenula (VHb), exiting where the dorsal habenula (DHB) is unaccompanied by its ventral counterpart. Injections limited to the VHb therefore often
label axons descending from the DHb (Table 3). Fibers originating from the VHb leave at more rostral and intermediate regions [fr(r); Figure 2: 7, 8; Figure 3i] where they form a thick fiber bundle. The fr(r) runs first lateral to the anterior thalamic nucleus (A), continuing between the anterior (A) and lateral (L) thalamic nucleus. These fibers merge partly with the ventral part of the fr(c) and compose the mantle portion of the bundle.

### 3.19 Ventral habenula efferents to the thalamus

Some scattered axons were found to run through the anterior thalamic nucleus and along the third ventricle. After VHb tracer injections few axonal appositions were observed in the central thalamic nucleus (C; Figure 3o; Table 3; n = 4). The VHb descending fibers make up a large part of the lateral/intermediate route and can first be differentiated in section 10 of Figure 2 based on their projection target and partial separation from the main tract.

### 3.20 Ventral habenula efferents to the ventral hypothalamus, preoptic area, septum, and nucleus of the diagonal band of Broca

A distinct plexus of VHb fibers aggregates at the fissure of the downward prolonging hypothalamus (Figure 3p). Few rostrally directed VHb fibers branch out and reach via the medial forebrain bundle (mfb) the ventral alar hypothalamus (Figure 2: 9; Figure 3n; Table 3; n = 4), preoptic area (Figure 2: 5; Figure 3g; Table 3; n = 6), medial and lateral septum (S; n = 5), Ndb (Figure 2: 4, 3; Figure 3c,e; Table 3; n = 6), and MOB (Figure 2: 1–2; Table 3; n = 4).

### 3.21 Ventral habenula efferents to the superficial mamillary area and posterior tubercle

In some cases, a more prominent single lateral branch originating from the VHb (habenulo-superficial-mamillary tract: hbsm) was observed, finally terminating in the superficial mamillary area (Sm; Figure 2: 10–12; Figure 3p; Table 3, n = 5). Complementary tracer injections into the Sm confirmed this observation (n = 6) and revealed additional projections to the dopaminergic and serotonergic systems, reassured by tracer applications into the posterior tubercle (n = 3) and raphe magnus (n = 3; data not shown). Further intermediate extending VHb fibers (habenulo-posterior-tubercle tract, hbp) concentrate around the superficial layer of the retromamillary area (Rm; n = 6) and the dorsomedial (TPdm; n = 6), and dorsolateral (TPd; n = 5) nucleus of the posterior tubercle, partly crossing to the contralateral hemisphere (Figure 2: 11, 12; Figure 3q). This result was validated by Neurobiotin application into TPdm/Rm (n = 4) or TPdm/dl (n = 6; data not shown). Some additional axons reach the deep densely packed layers of the retromamillary area (Rm; n = 5), the nucleus of the periventricular organ (Npv; n = 5), and histaminergic nucleus (Hist; n = 5; Figure 3q).

### 3.22 Ventral habenula efferents to the torus semicircularis and optic tectum

After tracer applications including the VHb, few efferent labeled fibers diverged from the fr (Figure 2: 13) and reach the torus semicircularis (Figure 2: 15; Figure 3u) and the cell-poor region around the third ventricle (Figure 2: 14; v; Table 3; n = 7). Fibers, which extend to the OT (sfgs, sgc, sgp; Figure 2: 14, 15; Figure 3t; Table 3; n = 5), can be found after tracer application in both DHb and VHb. The axons diverge early from the fr (Figure 2: 9, 10) and take a lateral course (optl).

### 3.23 Dorsal habenula efferents to the interpeduncular and interfascicular nucleus

Further DHb and VHb fibers that have remained in a more dorsal route, converge from the lateral side in a mediocaudal direction (Figure 2: 13). Three distinct bundles can be identified at the section 14 of Figure 2. The thin dorsal bundle terminates in the interfascicular nucleus (IF; Figure 3s; n = 6) and is therefore described as the habenulo-interfascicular tract (hbif). This tract originates from the DHb (Table 3), confirmed by complementary tracer applications (data not shown; n = 4). The habenulo-interpeduncular tract (hbip), also originating from the DHb (Table 3), remains in a further lateral course. The hbip forms a very dense neuropil pervading the whole interpeduncular nucleus, crossing the midline in a zigzag fashion (Figure 2: 16, 17; Figure 3w,x; n = 6). Injections into the right habenula resulted in dense fiber labeling of the IP at its rostral end [hbip(r); Figure 2: 16; Table 3], while injections into the left habenula also included the more caudal proportions [hbip(c); Figure 2: 17; Figure 3w; Table 3]. This can be attributed to connections by the left dorsolateral habenula (DlHb). The DHb projection to the IP was confirmed by complementary injections (data not shown; n = 3).

### 3.24 Ventral habenula efferents to locus coeruleus and laterodorsal tegmental nucleus

Dorsally to the IP, labeled axons were found in the locus coeruleus (LC) and laterodorsal tegmental nucleus (LDT; Figure 2: 17; Figure 3v; n = 5). These axons have their origin in the VHb (Table 3).

### 3.25 Ventral habenula efferents to the raphe nuclei

The habenulo-raphe tract (hrbr) runs ventral to the hbip and reaches the midline at a comparable level (Figure 2: 14–16). Injections limited to the DHb did not label the hrbr (Figure 3w; Table 3). Sparse labeling can also be seen dorsal to the IP in the superior central and dorsal raphe (RaCS-RaD) complex (Figure 2: 17; Figure 3x; n = 4) after injections including the VHb. The hrbr extends at the floor of the midbrain tegmentum, becoming less dense in the proceeding caudal direction and continues as far as the caudal end of the nucleus raphe magnum (RaM; Figure 2: 20; Figure 3y; n = 6). Neurobiotin application into the RaM at the level of the cerebellum labeled neurons in the entire VHb.
The present study provides evidence for the role of the habenula as an evolutionary conserved link between basal ganglia, limbic, and sensory systems. In lampreys, chondrichthyes, teleosts, amphibians, reptiles, and mammals, habenular afferents originate mostly from telencephalic, diencephalic, and mesencephalic areas [lampreys: Yáñez & Anadón, 1994, Stephenson-Jones, Floros, et al., 2012; chondrichthyes: Giuliani, Minelli, Quaglia, & Villani, 2002; teleosts: Villani et al., 1994, Yáñez & Anadón, 1996, Hendricks & Jesuthasan, 2007, Turner et al., 2016; amphibians: Kemali et al., 1980, present study; reptiles: Díaz & Puelles, 1992a; mammals: Herkenham & Nauta, 1977, McBride, 1981, Parent, Gravel, & Boucher, 1981]. During phylogenetic development, direct sensory innervation was gradually replaced by numerous inputs from limbic structures, as demonstrated for lampreys (Stephenson-Jones, Floros, et al., 2012), anuran amphibians (present study), and mammals (cf. Herkenham & Nauta, 1977). It was therefore argued that the context, in which the habenula circuitry is recruited might have changed (Stephenson-Jones, Floros, et al., 2012). Habenular efferents terminate predominantly in diencephalic, mesencephalic, and rhombencephalic areas, with an additional forebrain projection in tetrapods [lampreys: Yáñez & Anadón, 1994, Stephenson-Jones, Floros, et al., 2012; chondrichthyes: Giuliani et al., 2002; teleosts: Villani et al., 1994, Yáñez & Anadón, 1996, Amo et al., 2010; amphibians: Kemali et al., 1980, Laberge & Smith, 2017, present study; reptiles: Distel & Ebbesson, 1981, Díaz & Puelles, 1992b; mammals: Cragg, 1961, Akagi & Powell, 1968, Smaha & Kaelber, 1973, Herkenham & Nauta, 1979, Araki, McGeer, & Kimura, 1988]. In the course of vertebrate evolution, the primordial habenula circuitry seemingly underwent various evolutionary adaptations and the complexity of the afferent and efferent connectivity increased in the tetrapod line. It can therefore be hypothesized, that the habenula incorporated additional functions while maintaining the same functional principle. This would also account for the involvement of the habenula of various mammals in a variety of behaviors (cf. Hikosaka, 2010; Lecourtier & Kelly, 2007). Anuran amphibians, decedents of the first land-living tetrapods, seem to exhibit a mix of ancient as well as modern features of the habenula circuitry. The more complex network structure among tetrapods can therefore be considered as a secondary interconnection principle. In the following, arguments are provided which support a homology of the anuran DHb and VHb to mammalian MHb and LHb, respectively. This contradicts the assessment of Laberge and Smith (2017), who suggested that the conservation of the habenula circuitry among vertebrates is overestimated. Figure 5 displays the habenula circuitry in key positions during evolution. Based on available data, comparisons will be made to other anuran amphibians. Possible evolutionary adaptations are considered in comparison to lampreys, chondrichthyes, teleosts, reptiles, birds, and mammals. An overview of the function of afferent and efferent brain region is provided to contextualize habenula functions.

4.2 | Bed nucleus of the stria terminalis and ventral pallidum input to the ventral/lateral habenula

In the present study, retrogradely labeled neurons after Neurobiotin tracer application including the VHb were found in the ventral and dorsal part of the BST/VP at rostral levels, as well in the BST at caudal levels. This fits to the efferent connectivity of the BST in the rat (Dong & Swanson, 2004) as well as to retrogradely labeled cells in the VP after tracer application into the LHB (Herkenham & Nauta, 1977). Moreover, BST input to the habenula can also be found in the chicken, Gallus domesticus (Bálint, Mezey, & Csillag, 2011), indicating a common feature among tetrapods (Figure 5). In non-tetrapods, no homolog input has been reported, suggesting an increasingly complex network organization between limbic system and habenula during the phylogenetic development. In mammals, the BST mediates many behavioral responses to aversive or threatening stimuli [reviewed by Walker, Toufexis, and Davis (2003)], while the VP is important for reward and incentive motivation [reviewed by Smith, Tindell, Aldridge, and Berndige (2009)]. This is consistent with the role of the habenula of rats in governing the attribution of incentive motivational salience to reward predictive cues (Danna, Shepard, & Elmer, 2013).

4.3 | Possible striatal input to the habenula

In B. orientalis, few neurons in the striatum or striatopallidal system can be found after Neurobiotin tracer injection into the habenula (Table 2). The striatum is part of the basal ganglia and a critical component of the motor system. Following Neurobiotin application to the striatum, few fibers entering the habenula could be observed, but no axonal varicosities were found. In the rat, no evidence for direct striatal input is provided (Herkenham & Nauta, 1977). By contrast, in the river lamprey, Lampetra fluviatilis, striatal connections to the MHb homolog were reported (Stephenson-Jones, Floros, et al., 2012). Furthermore, in the Arabian carpetshark, Chiloscyllium arabicum, the presence of labeled neurons in the area periventricularis ventralis of the telencephalon was described (Giuliani et al., 2002), which, in the spiny dogfish, Squalus acanthias, exhibits histochemical similarities to the striatum (Northcutt, Reiner, & Karten, 1988). In the zebrafish, Danio rerio, fibers within the habenula might originate from cells in the dorsal subpallium (Rink & Vollm, 2004), which corresponds to a partial striatum homolog. The same could apply for labeled neurons in the posterior zone of the dorsal nucleus of the area ventralis (dorsal subpallium) in the goldfish, Carassius auratus (Villani et al., 1994). It might therefore be feasible, that striatal input to the habenula is a phylogenetically ancient trait, lost in tetrapods. Another explanation could be that striatal input of non-tetrapods coincides with the ventral striatum (nucleus accumbens) input of tetrapods, through exapta- tion of a preexisting loop.
FIGURE 5  Overview drawings showing the afferent and efferent connectivity of the lamprey (a), anuran (b), and mammalian (c) habenula. Purple lines indicate afferent brain areas, red lines indicate LHB (homolog) efferents, blue lines MHB (homolog) efferents. Dotted lines indicate efferents which cannot clearly be assigned to LHB or MHB homologs. The afferent habenula connectivity is based on lamprey: Yáñez and Anadón (1994), Stephenson-Jones, Floros, et al. (2012), Ocaña et al., 2015; amphibians: Present study; mammals: Herkenham and Nauta (1977), McBride (1981), Parent et al. (1981). The efferent habenula connectivity is based on: Lamprey: Yáñez and Anadón (1994), Stephenson-Jones, Floros, et al., 2012; amphibians: Present study; mammals: Cragg (1961), Akagi and Powell (1968), Smaha and Kaelber (1973), Herkenham and Nauta (1979), Araki et al. (1988). Brains (a,b) are redrawn after Nieuwenhuys, ten Donkelaar, and Nicholson (1998). For abbreviations, see list.
4.4 | Nucleus accumbens input to the ventral/lateral habenula

The description of habenula afferents from the nucleus accumbens is less clear among vertebrates. In mammals, the nucleus accumbens plays a significant role in incentive salience, aversion, and positive reinforcement (Saddoris, Cacciapaglia, Wightman, & Carelli, 2015; Wenzel, Rauscher, Cheer, & Oleson, 2015). In lampreys and chondrichthyes, no clear subdivision between the striatum (basal ganglia) and nucleus accumbens (limbic system) is reported. A separate “mesolimbic” and “nigrostriatal” projection might be a trait that developed later in phylogeny. O’Connell and Hofmann (2011) argued, that neurochemical and hodological evidence in teleost fish points to the dorsal area of the subpallium as the partial putative nucleus accumbens homolog. Following this argument, labeled cells in the anterior zone of the dorsal nucleus of the area ventralis telencephali in the goldfish (Villani et al., 1994) could also correspond to nucleus accumbens input to the habenula. As argued above for possible striatum input to the habenula in the zebrafish, labeled cells in the dorsal subpallium could also correspond to a partial nucleus accumbens homolog (cf. Rink & Wullimann, 2004) but no clear classification for teleosts can be made based on the currently available data. It is therefore conceivable, that this connection is not present in teleosts.

According to the present findings, labeled cells in the nucleus accumbens can only be found after injections including the VHb (Table 2; Figure 5), which replicates the connectivity of the LHb in the rat (Herkenham & Nauta, 1977). The connection to the habenula could also be observed in the African clawed frog, Xenopus laevis, and the Spanish ribbed newt, Pleurodeles waltli, after tracer injections into the ventromedial telencephalic wall (Marín, Smeets, & González, 1998b). Nucleus accumbens input to the habenula seems therefore a feature at least present in tetrapods.

4.5 | Pallium/cortex input to the ventral/lateral habenula

After tracer applications including the VHb, faintly labeled neurons and fibers were found in the ipsilateral rostral pallium (rP; Table 2; Figure 5). The anterogradely labeled axons after rP application follow the lateral corticohabenular tract (chht), as described in the early 19th century for the tiger salamander, Ambystoma tigrinum (Herrick, 1927). This tract was used in the northern leopard frog, Rana pipiens, and the American bullfrog, Rana catesbeiana, to describe a dorsal proportion of the lateral olfactory tract (Northcutt & James Royce, 1975; Scalia, Gallousis, & Roca, 1991; Scalia, Halpern, Knapp, & Riss, 1968). However, as revealed in the present study, axons of the rostral pallium also join this tract and innervate the VHb. It was argued, that the rP, 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 et al., 2007). In the rat, synaptic input from the prefrontal cortex (PFC; Figure 5) to the LHb could be demonstrated (Greatrex & Phillipson, 1982). Afferents from the PFC to the VHb in anurans seem therefore conceivable. Innervation of the habenula by the lateral pallium (Lp; Figure 5) in sea and river lampreys substantiates the palliohabenular projection as a phylogenetically early trait (Ocaña et al., 2015). The pallial/cortical projection to the habenula is also in accordance with other vertebrate species. For instance, in the Arabian carpetshark retrogradely labeled neurons in the pallium dorsale pars superficialis were reported after habenula tracer application (Giuliani et al., 2002).

Despite few discrepancies, circumstantial evidence points to a common pattern among vertebrates.

4.6 | Bed nucleus of the pallial commissure and prethalamic eminentia input to the habenula

Further afferent connections of the habenula in B. orientalis originate from the bed nucleus of the pallial commissure (BN) and prethalamic eminentia (PTE), with few cells in the ventral area possible corresponding to the bed nucleus of the stria medullaris. A reciprocal connection of the BN with the habenula was already demonstrated in the gray treefrog, Hyla versicolor (Endepols, Roden, & Walkowiak, 2005), while innervation by the prethalamic eminentia (thalamic eminentia in old nomenclature) is described for urodeles and anurans (Krug, Wicht, & Northcutt, 1993; Laberge & Roth, 2007). Studies in zebrafish reported input from a comparable cell formation in a posterior area of the pallium above the anterior commissure and the presumptive prethalamus (Turner et al., 2016). In the Tenerife lizard, Gallotia galloti, input to the habenula arises from the nucleus of the posterior pallial commissure, (pre-)thalamic eminentia, and nucleus of the stria medullaris (Díaz & Puelles, 1992a). Descriptions in the larval sea lamprey, Petromyzon marinus, are less clear, but these regions could correspond to labeled neurons in the subhippocampal lobe and the thalamus (Yáñez & Anadón, 1994). Input from corresponding homologous structures is not specified for mammals.

4.7 | Ventral habenula input from the ventral prethalamus, the homolog of the entopeduncular nucleus?

In mammals, primary input from the basal ganglia to the LHb (and not MHB) arises from the border region of the entopeduncular nucleus (EP), widely held to be homologous to the internal pallidal segment of primates (GPi; Herkenham & Nauta, 1977; Shabel, Proulx, Trias, Murphy, & Malinow, 2012). Although the EP is predominantly GABAergic, habenula input from this region is glutamatergic, excitatory, and aversive (Shabel et al., 2012). Another study described in the rat habenula additionally inhibitory and excitation-inhibition sequences, depended on the stimulation site in the dorsal or ventral EP (Garland & Mogenson, 1983). A homolog structure in anuran amphibians has yet to be identified. A promising region that requires more research was identified in this study. This is based on following arguments. (1) As argued by Turner et al. (2016), the entopeduncular nucleus is likely to be of diencephalic origin in all vertebrates. (2) In the lamprey, a glutamatergic habenula projecting pallidal nucleus is described.
(Stephenson-Jones, Ericsson, Robertson, & Grillner, 2012). (3) In the zebrafish, the habenula projecting entopeduncular nucleus originates developmentally from the diencephalic PTE, expressing both tbr1 and lhx5 (Amo et al., 2010; Turner et al., 2016). (4) In X. laevis, the prethalamic eminence expresses tbr1 whereas the prethalamus expresses lhx5 (Brox, Puelles, Ferreiro, & Medina, 2004; Domínguez, Morona, González, & Moreno, 2013; Moreno et al., 2004). (5) In the present study, the primary input region of the VHb (and not DHb) can be found in the ventral boundary of the prethalamus (Mv, VLv, and partly Ep). Previous anatomical studies described comparable labeling for the edible frog, Rana esculenta, and B. orientalis following habenula tracer application (Kemali et al., 1980; Roth et al., 2007). (7) In Tenerife lizard, strong habenula input originates from the ventrolateral (pre-)thalamic nucleus and anterior entopeduncular nucleus (Diaz & Puelles, 1992a), comparable in location with the anuran Mv, VLv, and Ep. (8) In the adult X. laevis, the ventral (pre-)thalamus is positive for xGAD67, the GABA catalyzing enzyme (Brox, Puelles, Ferreiro, & Medina, 2003). (9) Additionally, this area exhibits glutamatergic neurons (Maier et al., 2010). (10) As the mammalian entopeduncular nucleus, the anuran ventral (pre-)thalamus and Ep receive input from the striatum (Wilczynski & Northcutt, 1983; Marin, Smeets, & González, 1997b), while the Ep possibly projects to the pedunculopontine nucleus and anterior thalamic nucleus (cf. Maier et al., 2010). Circumstantial evidence points in anurans to a promising candidate structure for a homolog of the rodent entopeduncular nucleus (i.e., Ep), as well as a habenula-projecting part (i.e., Mv, VLv).

However, the developmental origin of the entopeduncular nucleus of rodents is presently controversial. Puelles, Martinez-de-la-Torre, Bardet, and Rubenstein (2012) argue, that the mouse entopeduncular nucleus has an alar hypothalamic origin. Due to the topographical location of the labeled cells in this study and the ambiguous data on development markers, this region should be examined more closely in future studies. Yet, it is possible that basal ganglia input to the habenula is a common feature among vertebrates.

4.8 | Reciprocal connection of the habenula with the septum and nucleus of the diagonal band of Broca

Limbic input to the habenula in B. orientalis arises from the septum (S) and the nucleus of the diagonal band of Broca (Ndb; Figure 5). This septal projection was already described in R. esculenta (Kemali et al., 1980) and in more detail, including the Ndb projection, in H. versicolor (Endepols et al., 2005). A differential input of DHb and VHb could not be determined, which is in accordance with the description of the afferent connections of MHb and LHB in the rat (Herkenham & Nauta, 1977; Mok & Mogenson, 1972), as well as in the cat and monkey (Parent et al., 1981). Whereas septal input is not described in sea lampreys, it could correspond to labeled cells in a rostral area of the subhippocampal lobe (Yáñez & Anadón, 1994). There is still the possibility that septal input is a more recent phylogenetic development (Stephenson-Jones, Floros, et al., 2012). In the Arabian carpetshark input to the habenula arises from the area septi (Giuliani et al., 2002). In the zebrafish, comparable input originates from the ventral area of the subpallium (Turner et al., 2016), which is interpreted as the homolog of the septum (Rink & Wullimann, 2004). The presence of septo-habenular projections in teleost is also in agreement with the description of similar telencephalic input in the rainbow trout, Oncorhynchus mykiss, (Yáñez & Anadón, 1996) and the goldfish (Villani et al., 1994) even though—because of the limited anatomical information available—some of these connections could also correspond to afferents from the striatum. A comparable situation can be found in the Tenerife lizard where additional input from diagonal band nucleus is described (Díaz & Puelles, 1992a). Septo-habenular input is also characterized for the gold tegu, Tupinambis nigrpunctatus, (Sligar & Voneida, 1981) and the pigeon (Krayniak & Siegel, 1978). The septo-habenular projection is therefore a feature, which is at least present in tetrapods, while the description in chondrichthyens and teleosts is ambiguous.

Additionally, the septum and nucleus of the diagonal band in B. orientalis is innervated by the VHb, which is in agreement with LHb projections of mammals (Akagi & Powell, 1968; Araki et al., 1988; Herkenham & Nauta, 1979; Tomimoto, Kamo, Kameyama, McGeer, & Kimura, 1987) and reptiles (Díaz & Puelles, 1992b; Distel & Ebbesson, 1981). As in mammals and reptiles, these fibers diverge from the fasciculus retroflexus and reach the forebrain via the medial forebrain bundle. The LHb/VHb is therefore reciprocally connected with the septum and Ndb, suggesting the existence of a complex feedback loop. This supports an increasingly complex habenula circuitry in tetrapods. Lesion experiments showed that the septum has an inhibitory control over sexual behavior in anurans (Walkowiak, Berlinger, Schul, & Gerhardt, 1999), snakes (Krohmer & Crews, 1987a), songbirds (Goodson, Eibach, Sakata, & Adkins-Regan, 1999), and mammals (Tsukahara & Yamanouchi, 2001). These connections may be crucial for the involvement of the habenula in sexual behavior, as demonstrated in mammals (reviewed by Sutherland, 1982).

4.9 | Reciprocal connection of the habenula with the preoptic area

Additional limbic projections to the habenula arise from the preoptic area (Poa) and the suprachiasmatic nucleus (Sc) in B. orientalis. In the rat, Herkenham and Nauta (1977) describe the lateral preoptic-hypothalamic continuum as the second major afferent connection of the LHB. They argue that moderate numbers of fibers originating from the lateral preoptic area by-pass the MHb via the stria medullaris. No such distinction could be made in the present study. Small deposits of Neurobiotin into the DHb and VHb equally stained neurons in the preoptic area, even when labeling of the stria medullaris was excluded (Table 2). Only injections including the VHb occasionally labeled neurons in the suprachiasmatic nucleus. For the Arabian carpetshark, labeled neurons in the nucleus preopticus are reported (Giuliani et al., 2002). In the zebrafish, few scattered cells in the preoptic region could be observed (Turner et al., 2016); while in the rainbow trout, Yáñez and Anadón (1996) described sparsely labeled neurons along the preoptic nucleus. In the Tenerife lizard, strong projections to the...
habenula have their origin in the lateral preoptic area (Diaz & Puelles, 1992a).

Furthermore, in mammals (Akagi & Powell, 1968; Araki et al., 1988; Herkenham & Nauta, 1979) and reptiles (Distel & Ebbeson, 1981), few LHb fibers reach the preoptic area, suggesting a reciprocal connection. This is following own data in B. orientalis and was discussed as a potential connection by Laberge and Smith (2017). This habenulo-forebrain loop is, therefore, at least conserved in tetrapods. The preoptic area is related to sexual, courtship, and thermoregulatory behavior in anurans (Bicego & Branco, 2002; Knorr, 1976; Schmidt, 1984, 1989), songbirds (Mills & Heath, 1972; Riters & Ball, 1999), snakes (Friedman & Crews, 1985; Krohmer & Crews, 1987b), and mammals (Heimer & Larsson, 1967; Nagashima, Nakai, Tanaka, & Kanosue, 2000). The involvement of the habenula of rats in maternal behavior may be explained by this connection [reviewed by Sutherland (1982) and Hikosaka (2010)].

4.10 | Reciprocal connection of the habenula with the hypothalamus

In B. orientalis numerous labeled cells following DHb and VHb tracer injection were found in the dorsal and ventral hypothalamus (Figure 5), which is in in compliance with previously established lateral hypothalamus (LH) input to the LHb (homolog) in mammals (Herkenham & Nauta, 1977; Parent et al., 1981), reptiles (Diaz & Puelles, 1992a), and lampreys (Stephenson-Jones, Floros, et al., 2012). The literature contains only fragmentary accounts of possible comparable habenula input in teleosts (Turner et al., 2016; Yáñez & Anadón, 1996) and sharks (Giuliani et al., 2002). Additionally, some or all of these connections might be assigned to projections of the posterior tubercle.

In the present study, few VHb (and not DHb) fibers diverged from the fasciculus retroflexus and reached via the medial forebrain bundle the rostral ventral hypothalamus. This is in accordance with the description of LHb (and not MHB) efferents in the cat, opossum, and rat (Akagi & Powell, 1968; Araki et al., 1988; Herkenham & Nauta, 1979; Smaha & Kaelber, 1973), as well as to the projection of the fasciculus retroflexus in the Tenerife lizard (Diaz & Puelles, 1992b). In B. orientalis, an extensive overlap of axonal projections between VHb and DHb was suggested, including the projection to the alar and tuberal hypothalamus. It was argued that these axonal targets are unique among vertebrates and present a problem for a plausible scenario of the phylogenetic conservation of the habenula circuitry (cf. Laberge & Smith, 2017). However, these observations can also be explained by connections to the posterior tubercle and the ventral tegmentum, and therefore do not contradict the evolutionary conservation of the habenula circuitry. Nevertheless, the tetrapod habenula seems to have a reciprocal connection with a limbic area involved in the regulation of food intake, energy balance, and other autonomic functions (cf. Berthoud & Munzberg, 2011). Correspondingly, the habenular nuclei were described to regulate cardiovascular activities during insular epilepsy (Lv, Ma, Meng, Li, & Lin, 2012).

4.11 | Reciprocal connection of the habenula with the torus semicircularis and optic tectum

The results of this study show some habenula input arising from the superior colliculus (SC) and inferior colliculus (IC) homologs, the optic tectum (OT), and torus semicircularis (Ts), thus relaying sensory information to the habenula. Early anatomical evidence also points in teleost fish to the presence of tecto-habenular fibers (Schnitzlein, 1962). In the lamprey direct input from the pretectum to the MHB homolog can be found (Capantini, von Twickel, Robertson, & Grillner, 2017; Stephenson-Jones, Floros, et al., 2012), which could correspond to input from the pretectal nucleus of the posterior commissure (Npc) identified in this study. Comparable input is not described in mammals. Sensory input seems therefore to be a feature only present in anamniotes.

Additionally, in the present study anterograde labeled fibers were observed in the OT and Ts. This reciprocal connection is congruent with early observations of Herrick (1948) about the brain of the tiger salamander, A. tigrinum. Projections to sensory systems can also be found in other tetrapods. Thus, autoradiographic, fiber degeneration, and viral PHA-L anterograde tracing experiments seem to substantiate the habenula projection to the SC in the rat, cat, and opossum (Akagi & Powell, 1968; Araki et al., 1988; Herkenham & Nauta, 1979; Way & Kaelber, 1969). Furthermore, fiber degeneration studies revealed habenula efferents to the IC in the cat (Akagi & Powell, 1968) and opossum (Way & Kaelber, 1969).

It seems that the Ts also gives rise to spinal projections, mainly from its laminar nucleus (Walkowiak & Luksch, 1994). It is therefore argued, that at least part of this projection may correspond to the periaqueductal gray (PAG) of mammals (Sánchez-Camacho, Marín, ten Donkelaar, & González, 2001). The present observation could therefore also correspond to the reciprocal connection of the central ventral gray (or PAG) with the LHb in the rat (Herkenham & Nauta, 1977, 1979). A separate PAG could therefore be an exaptation of a pre-existing circuitry.

The OT/SC plays in vertebrates—inter alia—an important role in goal-directed movements (reviewed by Grillner, Wallén, Saitoh, Kozlov, & Robertson, 2008) and the PAG in rats in the acquisition and execution of defensive behaviors (De Oca, DeCola, Maren, & Fanselow, 1998). The projection of the habenula to these regions may be crucial for habenula involvement in place conditioned aversion and aversion-driven escape behaviors (Lecca et al., 2017; Terenzi, Guimaraes, & Prado, 1990; Thornton & Evans, 1982).

4.12 | Olfactory fibers bypassing the habenula and habenula projection to the olfactory bulbs

In amphibians, the olfactory bulbs and their associated tracts (Figure 5: 1–7) do not innervate the habenula, but rather cross via the habenula commissure to the contralateral hemisphere, as revealed by early fiber degeneration and horseradish peroxidase (HRP) anterograde tracing studies in R. catesbeiana and R. pipiens (Northcutt & James Royce, 1975; Scalia, 1976; Scalia et al., 1991). As in these
studies, no evidence of olfacto-habenular fibers in *B. orientalis* could be found. Endorsing this, efferents of the olfactory bulbs in the lizard, snake, and pigeon cross via the habenula commissure to the contralateral side (Lanuza & Halpern, 1998; Martínez-García, Olucha, Teruel, Lorente, & Schwerdtfeger, 1991; Rieke & Wenzel, 1978). In the rat, retrogradely labeled cells in the olfactory tubercle were assigned labeling of fibers of passage, which project via the stria medullaris to the subjacent mediadorsal thalamic nucleus (Heimer, 1972; Herkenham & Nauta, 1977).

By contrast, in lampreys, olfactory bulb neurons projecting to the MHB homolog were described (Stephenson-Jones, Floros, et al., 2012), while in zebrafish spatially organized odor responses in the DHb (MHB homolog) were proposed to gate the olfactory information to DHB targets (Jetti, Vendrell-Llopis, & Yaksi, 2014). Additionally, Turner et al. (2016) stated asymmetric afferents from the olfactory bulb to the right DHb. Thus, it is possible that projections of the olfactory bulbs to the habenula were lost in tetrapods. This illustrates the shift from direct sensory to limbic input.

Additionally, the MOBs in *B. orientalis* seem to be innervated by the VHB, as demonstrated in the present study. Correspondingly, Roth et al. (2004) described habenula projections to the MOBs in *B. orientalis*. This reflects the early anatomical evidence of a projection from the LHb to the olfactory tubercle (ot) in cat and opossum (Akagi & Powell, 1968; Way & Kaelber, 1969) or to the substantia innominata (which includes the olfactory tubercle) in the rat (Araki et al., 1988; Herkenham & Nauta, 1979). The habenula could therefore be involved in the modulation of sensory systems. Accordingly, habenula lesioned rats failed in odor discrimination tasks at low concentrations (Rausch & Long, 1974).

### 4.13 Thalamic fibers bypassing the habenula and habenula projection to the thalamus

While thalamic input to the habenula has recently been under discussion—based on early data in reptiles, anuran amphibians, and teleost (Jesuthasan, 2017)—many of these proposed thalamic regions are now described as part of the prethalamus. In the current study, injections into the habenula often labeled cells in the anterior (A), lateral (L), and central (C) thalamic nucleus, which extend their axons laterally along the stria medullaris. Because the number of retrogradely labeled cells increased with an increase of stria medullaris labeling, it can be assumed that these cells can be attributed to fibers of passage. In *B. orientalis*, after VHB tracer injection few fibers reach the central thalamic nucleus (Figure 5), a feature also described for the ventromedial thalamus (VM) in mammals and reptiles (Akagi & Powell, 1968; Araki et al., 1988; Distel & Ebesson, 1981; Herkenham & Nauta, 1979).

### 4.14 Amygdala fibers bypassing the habenula

In *B. orientalis*, moderate to abundant cell labeling could be observed in the medial (MeA), lateral (LA), and central extended (CeA) amygdala after habenula tracer injections (Table 2). In the rat, retrogradely labeled neurons belong to amygdalo-fugal fibers in the stria medullaris that do not extend to the habenula and which are actually connections to the mediadorsal thalamic nucleus (Herkenham & Nauta, 1977). Accordingly, an older electrophysiological study in the rat failed to demonstrate a direct amygdala input to the habenula (Mok & Mogensen, 1974b). Based on the data presented, it is uncertain whether this applies to the amygdalo-fugal fibers in anuran amphibians as well. However, in the anuran species *Rana perezi* and *I. laevis*, injections in the MeA resulted in labeled fibers in the stria medullaris, partly crossing the habenular commissure. Most of these fibers formed a dense terminal field in the contralateral habenula (Moreno & González, 2003). Still, these findings could also be explained by fibers bypassing the MeA, originating perhaps from the caudal BST or rostral pallium. Because most labeled cells in the present study can only be found in injections including the rostral stria medullaris or the habenula commissure, it seems likely that marked cells can be explained by fibers of passage and not by amygdala fibers terminating in the habenula. Another explanation could be that amygdala input of anurans coincides with striatal input of agnathans or limbic input of mammals, because in the tetrapod line the striatopallidalum of early vertebrates becomes the extended amygdala, consisting of centromedial amygdala connected with the bed nucleus of the stria terminalis (Loonen & Ivanova, 2016).

### 4.15 Fibers bypassing from and to the frontal organ/pineal complex

As with other tracers, we cannot exclude the possibility that Neurobiotin may also label fibers of passage. This could prove to be problematic because the frontal organ of anuran amphibians is also connected to the hypothalamus, preoptic area, pretectal area, and the lateral parts of the midbrain central griseum. The caudal root of frontal nerve is described to pass through the habenula commissure (Eldred, Finger, & Nolte, 1980; Kemali & De Santis, 1983; Zilles & Nickeleit, 1979). However, if the projection of the VHb to the hypothalamus, preoptic area, or other areas could be attributed to projections of the frontal organ, especially tracer applications omitting the DHb and habenula commissure should not label these fibers projections. This is not the case (see Table 3). Additionally, these studies also describe a different course of fiber projection. Furthermore, in the Mongolian gerbil, *Meriones urguiculatus*, efferent projections from the lateral geniculate nucleus to the pineal complex are described (Mikkelsen, Cozzi, & Møller, 1991). In *B. orientalis*, labeled cells in the lateral geniculate can especially be found after injections including the stria medullaris or habenula commissure. We attributed these connections therefore to the pineal complex.

### 4.16 Dorsal habenula efferents to the interpeduncular nucleus

The efferent connections of the anuran DHb are generally similar to those reported in the literature for DHb/MHb of other vertebrates (Figure 5). These include outputs through the habenula-
interpeduncular tract to the interpeduncular nucleus, a connection also conserved in lampreys, teleosts, reptiles, and mammals (Bianco & Wilson, 2009; Distel & Ebbesson, 1981; Herkenham & Nauta, 1979; Kemali et al., 1980). In lamprey and zebrafish, the asymmetric MHB homolog projects differentially to the interpeduncular nucleus (Bianco & Wilson, 2009; Stephenson-Jones, Floros, et al., 2012). The present study could provide comparable results, with a rostro-caudal asymmetry of projection, contradicting the dorsoventral asymmetry described by Laberge and Smith (2017). It is hypothesized that these asymmetries serve to increase control over behaviors in binary opposition like freezing versus escaping in anti-predator responses (Ichijo, Nakamura, Kawaguchi, & Takeuchi, 2017).

4.17 | Habenula efferents to the anuran VTA and SNc

The dopaminergic innervation of the striatum and nucleus accumbens in anuran amphibian arise primarily from distinct retromamillar, posteri- rior tubercular, and mesencephalic cell groups (Marín, González, et al., 1997a, 1997b; Marín, Smeets, & González, 1997b; Marín, Smeets, et al., 1997a; Marín et al., 1998a, 1998b). In mammals, direct projections of the LHb to the VTA and SNc (Akagi & Powell, 1968; Araki et al., 1997a, 1997b; Marín et al., 1998a, 1998b). In the present study, substantial habenula projections to the nucleus raphe magnus. A previous study in X. laevis investigated the hodological features of the tuberal and mamillary regions (Domínguez et al., 2014). Most of the described connections also fit the connectivity of the mammalian RMTg, including connection with the nucleus accumbens, preoptic area, septum, amygdala, bed nucleus of the stria terminalis, hypothalamus, and mesencephalic tegmentum (Jhou et al., 2009). Therefore, the superficial mamillary area in terms of its connectivity—may resemble the mammalian RMTg. The LHb-RMTg circuitry is believed to contribute to reinforcement learning and other cognitive functions by inhibition of serotonergic and dopaminergic transmission toward the forebrain [reviewed by Lecourtier & Kelly (2007) and Hikosaka (2010)].

4.18 | Ventral habenula efferents to the superficial mamillary area/RMTg

One of the main LHb connections that were discussed in recent years is the projection to the GABAergic RMTg, even if the habenular pro- jections are much more numerous, many of them appear to have slipped into oblivion. In anuran amphibians, no homologous structure is described so far. In the present study, we could demonstrate that the superficial mamillary area is innervated by the VHB. The superficial mamillary area is as well connected with the TPdm/dl and the nucleus raphe magnus. A previous study in X. laevis investigated the hodological features of the tuberal and mamillary regions (Domínguez et al., 2014). Most of the described connections also fit the connectivity—may resemble the mammalian RMTg. The LHb-RMTg circuitry is believed to contribute to reinforcement learning and other cognitive functions by inhibition of serotonergic and dopaminergic transmission toward the forebrain [reviewed by Lecourtier & Kelly (2007) and Hikosaka (2010)].

4.19 | Ventral habenula efferents to the serotonergic raphe nuclei

In the present study, substantial habenula projections to the nucleus raphe magnus emerged from the VHB. Less distinct was the projection to the superior central and dorsal raphe complex. The nomenclature is based on Adli et al. (1999) and assumes that the nuclei in frog are homolog to their mammalian counterparts. In mammals, connections to the dorsal (RaD) and medial (or superior central; CaCS) raphe nuclei are described (Akagi & Powell, 1968; Herkenham & Nauta, 1979; Smaha & Kaelber, 1973; Way & Kaelber, 1969) and in one study even projections to the nucleus raphe pontis (Araki et al., 1988). LHb fibers extend as far caudally as the inferior olivary nucleus (Smaha & Kaelber, 1973), which reflects the situation in the present study. The anuran nucleus raphe magnus could consequential correspond to the nucleus raphe pontis of mammals. Nevertheless, these observations are congruent with connections of the LHb (homolog) to the raphe.
nuclei in reptiles (Diaz & Puelles, 1992b; Distel & Ebbesson, 1981), teleosts (Yáñez & Anadón, 1996), and lampreys (Yáñez & Anadón, 1994). In the zebrafish, LHb homolog fibers in the median raphe were mainly observed in close association with glutamatergic neurons and only scarcely within GABAergic and 5-HT neurons (Nathan, Ogawa, & Parhar, 2015). Stephenson-Jones, Floros, et al., 2012, on the contrary, described for the lamprey connections to serotonergic neurons in the ventral mamillary area (vMAM), which should be in be in accordance with connections to the nucleus of the periventricular organ observed in B. orientalis. As in the present study, habenula input arises in rats from the raphe (i.e., nucleus raphe pontis and superior central raphe nucleus; Herkenham & Nauta, 1977).

5 | CONCLUSION

Anuran amphibians seem to exhibit a mix of ancient as well as modern features of the habenula circuitry: in contrast to lampreys, the anuran habenula exhibits strong interconnectivity with the limbic system and receives, in contrast to mammals, also input from sensory systems. In the course of evolution, the primordial habenula circuitry underwent various evolutionary adaptations and in the tetrapod line, the circuit complexity increased. Whereas this study presents evidence for the existence of complex habenula feedback loops with basal ganglia, limbic, and sensory systems, the exact functional consequences of this anatomical structure have yet to be determined by future studies. Hikosaka (2010) proposed that the habenula evolved as a general motor suppression system that was originally devoted to circadian control of behavior. This seems to be mediated via the indirect LHb-RMTg-VTA/SNc/raphe pathway. However, numerous connections with telencephalic, diencephalic, mesencephalic, and brainstem regions, as, for example, demonstrated in this study, remain neglected. The present study, therefore, could provide supportive evidence for a much more intricate interconnection principle throughout the vertebrate phylum. Thus, functional consequences were shown in an older electrophysiological study, Mok and Mogenson (1974a) could demonstrate in the rat excitatory and inhibitory input after habenula stimulation in many midbrain and brainstem regions, including the superior colliculus, pretectum, red nucleus, VTA, central gray (periaqueductal gray), and reticular formation. Functional aspects of the anuran habenula loops will be subject of a follow-up paper.

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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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