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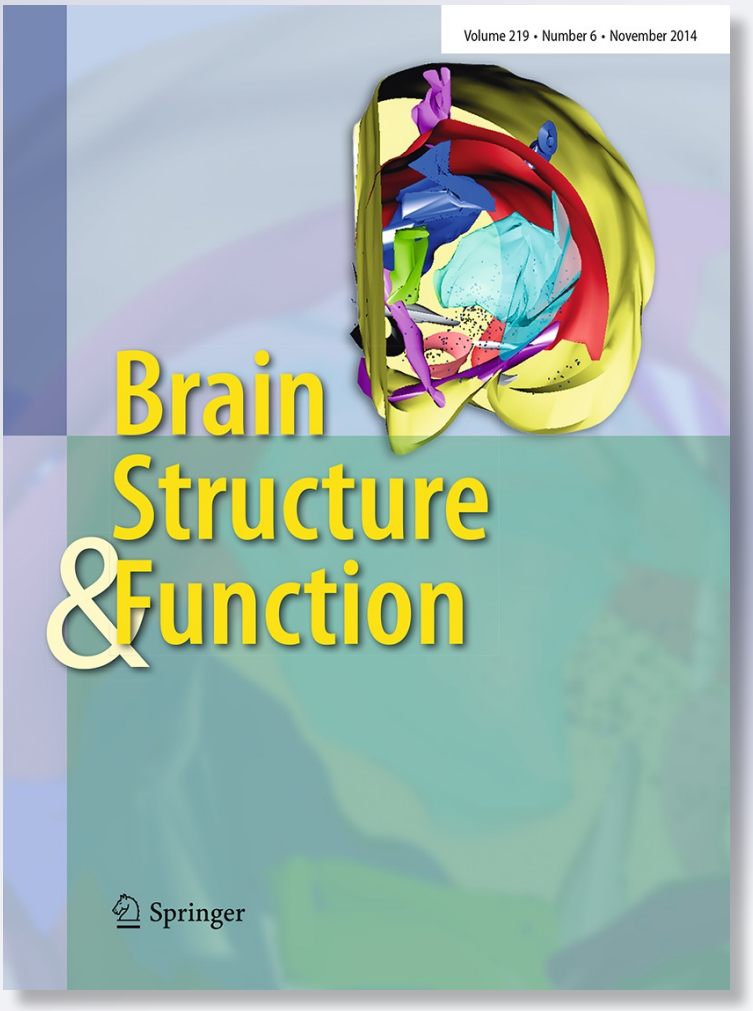

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ORIGINAL ARTICLE

Organization of afferents to the striatopallidal systems in the fire-bellied toad *Bombina orientalis*

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Abstract The cerebral hemispheres of amphibians display paired dorsal and ventral striatum (commonly referred to as striatum proper and nucleus accumbens, respectively). Each striatal region is proposed to be closely associated with a pallidal structure located caudal to it to form a striatopallidal system. In the present study, afferents to the dorsal and ventral striatopallidal systems of the fire-bellied toad (*Bombina orientalis*) were investigated using the neuronal tracer biocytin. A quantitative analysis of the topographical distribution of afferent neurons from the thalamus and posterior tubercle/ventral tegmentum was emphasised. The main results show that inputs to the two striatopallidal systems originate from distinct dorsal thalamic nuclei, with dorsal and ventral striatopallidal afferent neurons favouring strongly the lateral/central and anterior thalamic nuclei, respectively. However, afferent neuron distribution in the dorsal thalamus does not differ in the rostrocaudal axis of the brain. Afferent neurons from the posterior tubercle and ventral tegmentum, on the other hand, are organised topographically along the rostrocaudal axis. About 85 % of afferent neurons to the dorsal striatopallidal system are located rostrally in the posterior tubercle, while 75 % of afferent neurons to the ventral striatopallidal system are found more caudally in the ventral tegmentum. This difference is statistically significant and confirms the presence of distinct mesostriatal pathways in an amphibian. These findings demonstrate that an amphibian brain displays striatopallidal systems integrating parallel streams of sensory information potentially under the influence of distinct ascending mesostriatal pathways.

Keywords Amphibian · Basal ganglia · Striatum · Pallidum · Biocytin

Introduction

The cerebral hemispheres of mammals comprise topographically organised projection systems made up of cortex, striatum and pallidum that target motor systems in the brainstem and engage the thalamus through collateral projections (Heimer 2003; Swanson 2005). Striatum and pallidum are parts of the cerebral nuclei found under the cortex. Together, these structures are sometimes referred to as the basal ganglia. Cortico-striatopallidal systems are organised for effective control of downstream brain regions that initiate and guide behaviour. There is a disagreement as to whether the cortical structures associated with the extended amygdala and septum/diagonal band should be regarded as independent cortico-striatopallidal systems or not, but general similarities in organisation are acknowledged and different opinions on the nature of cerebral hemisphere divisions are a matter of emphasis of regional differences over similarities (Heimer and Van Hoesen 2006).

A general pattern of striatopallidal organisation was recently demonstrated in the cerebral hemispheres of a basal vertebrate, the river lamprey *Lampetra fluviatilis* (Stephenson-Jones et al. 2011). Lampreys appear to possess only one such system suggested to play an important role in action selection, while multiple systems for major classes of motor responses and behaviours are proposed for

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mammals (see Swanson 2005). These findings suggest the possibility that multiple cortico-striatopallidal systems could have evolved through duplication/differentiation during vertebrate phylogeny. A test of this idea will require detailed description of the organisation of the cerebral hemispheres and their target regions in additional vertebrate groups. The present study aims to contribute to such a description of the striatopallidal systems in an anuran amphibian, the fire-bellied toad *Bombina orientalis*.

Amphibians present an interesting case of cerebral hemisphere organisation because their dorsal striatopallidal system receives very little input from the pallium (homologue of the cortex of amniotes) compared to mammals (Wilczynski and Northcutt 1983; Marín et al. 1997a; Roth et al. 2007). Strong pallial output is however directed to the medial subpallium, where a ventral striatopallidal system has been tentatively located (Marín et al. 1998a; Roth et al. 2004; Mühlenbrock-Lenter et al. 2005). The concept of striatopallidal system in amphibians was articulated by Endepols et al. (2004a) based on connectional and neurochemical data in four species of anurans. It posits rostral, intermediate and caudal parts in the ventrolateral telencephalic walls, where the rostral part is the main recipient of information while the intermediate and caudal parts are the output regions. These authors proposed that the rostral striatal parts are homologues of the mammalian dorsal striatum (or striatum proper), while the caudal parts are homologues of the dorsal pallidum. This dorsal striatopallidal system features strong local processing as seen by intense reciprocal projections between the dorsal striatum and pallidum as well as downstream outputs in position to influence behaviour. Following this model, work in the fire-bellied toad suggested that such organisation into a locally integrated striatopallidal system could also be seen ventromedially in the telencephalon, with the nucleus accumbens at its rostral pole and a ventral pallidum located caudal to it (Roth et al. 2004; Mühlenbrock-Lenter et al. 2005).

Differential input to the amphibian ventrolateral and ventromedial telencephalon is not limited to the pallium. Marín et al. (1997a) showed that different nuclei in the amygdala, dorsal thalamus, ventral parts of the diencephalon and midbrain as well as the parabrachial nucleus all innervate preferentially or exclusively one of the two striatal divisions. Recently published details of dorsal thalamic neuron anatomy in the fire-bellied toad confirmed that their ascending axonal projections are segregated to the ventrolateral or ventromedial telencephalon (Laberge et al. 2008). Striatal afferents from the posterior tubercle (diencephalon) and ventral tegmentum (midbrain) were detected in two species of anurans (*Rana perezi* and *Xenopus laevis*) and one species of urodele (*Pleurodeles watl*) by Marín et al. (1995, 1997a, b). The presence of a catecholaminergic marker in subsets of neurons displaying differential projections to the two striatal regions led the authors to suggest that amphibians possess both ventral (mesolimbic) and dorsal mesostriatal dopaminergic pathways ascending to the telencephalon (Marín et al. 1997b). Interestingly, no evidence of a mesocortical pathway has been found in anuran amphibians.

Despite the fact that neurons projecting to both striatal divisions are found in both the posterior tubercle and the ventral tegmentum of amphibians, the studies of Marín et al. did not provide a quantitative analysis to support their claim of distinct mesostriatal pathways. Neuron groups at the origin of mesostriatal pathways are arranged in a continuum rather than in segregated populations and there is much variability in topographical relationships of the neuron groups contributing to different mesostriatal pathways among tetrapods (Fallon and Moore 1978; Marín et al. 1998b). Indeed, the putative homologies of amphibian brain regions with the nuclei giving rise to the mammalian mesostriatal pathways (ventral tegmental area and substantia nigra) remain unresolved (O'Connell and Hoffman 2011). For example, the posterior tubercle could be homologous to the ventral tegmental area, the substantia nigra, or both. It is thus possible that a division between ventral tegmental area and substantia nigra is a special characteristic of amniotes (Yamamoto and Vernier 2011).

The topographical organisation of afferents to the striatopallidal systems has never been studied quantitatively in an amphibian. The present study used retrograde and anterograde biocytin tract tracing to describe afferents to the dorsal and ventral striatopallidal systems in the fire-bellied toad, an anuran that occupies a phylogenetical position close to the base of the anuran tree (Vitt and Caldwell 2009). A quantitative analysis of the topography of thalamic and posterior tubercle/ventral midbrain afferents was emphasised to test the validity of the concept of dual mesostriatal pathways in amphibians and compare topographical relationships of the two main groups of striatopallidal afferents.

Materials and methods

Animals

Forty-two adult fire-bellied toads of mixed genders were used in the present study. The animals were bought from a local supplier (National Reptile Supply, Mississauga, ON, Canada) and held at a temperature of 21 °C under a photoperiod of 12:12-h light:dark (lights on at 7:00 h). The toads were housed in groups in glass tanks (37 × 22 × 25 cm) with gravel substrate, broken clay pots, and flat stones for cover. They had continuous access

to water and were fed crickets (*Acheta domesticus*) lightly dusted with calcium and vitamin powder ad libitum once weekly. The experimental procedures were approved by the University of Guelph animal care committee under the guidelines of the Canadian Council on Animal Care.

Procedures

All experiments were carried out in vitro in isolated brain preparations. After deep anaesthesia by immersion in a solution of 0.5 % tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA), the animals were quickly decapitated, the lower jaw was removed, and the skull was opened from the roof of the mouth to enable brain dissection. The dissection was performed in Ringer's solution consisting of Na^+ 129 mM, K^+ 4 mM, Ca^{2+} 2.4 mM, Mg^{2+} 1.4 mM, Cl^- 115 mM, HCO^{3-} 25 mM, glucose 10 mM, bubbled with 95 % O^2/5 % CO^2 until a pH of 7.3 was achieved. Tract tracing of neural pathways was achieved by application of biocytin crystals (Sigma-Aldrich B4261, St. Louis, MO, USA) directly to the lightly lesioned surface of the brain outside of the Ringer's bath. In most cases, the brain was split longitudinally to allow two applications per animal. For retrograde labelling of afferent neuron somata, the lateral surface of the brain was approached, while the medial surface was approached for anterograde labelling of axon projections. Intact brains were used in some retrograde labelling experiments to additionally assess contralateral afferents. After biocytin crystal application, the brains were stored in oxygenated Ringer's solution for 5–6 h at room temperature and then at 4 °C overnight. On the next day, the brains were fixed in 2 % paraformaldehyde and 2 % glutaraldehyde, and then 50-μm-thick transverse sections were cut on a VT1200 vibrating microtome (Leica Biosystems, Wetzlar, Germany). Biocytin was visualised by means of an avidin–biotin horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine (Sigma) as chromogen with heavy metal intensification achieved by adding 0.03 % nickel sulphate and cobalt chloride to the solution (Adams 1981). Sections were lightly counterstained with cresyl violet, dehydrated in ethanol, cleared in xylene, and coverslipped before examination under the microscope.

Analysis

The assessment of biocytin application sites, retrograde labelling and axonal targets was done using a DM1000 light microscope (Leica). Photomicrographs were scanned using an Eclipse 90i upright microscope (Nikon, Tokyo, Japan) equipped with a Retiga 2000R digital camera (QImaging, Surrey, BC, Canada), modified and optimised for presentation using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA). Analysis of the topography of striatopallidal afferents involved counting labelled neurons on every brain section covering the diencephalon to the caudal midbrain. Only neurons of a full diameter were counted to avoid double counts, but otherwise no corrections were made. This quantitative analysis was limited to the rostrocaudal axis of the brain, as changes in brain shape along this axis made the comparison of mediolateral and dorsoventral positions of labelled neurons impractical. Statistical analyses and graphs were done in Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA).

Two different neuroanatomical frameworks have been used by investigators of the anuran diencephalon and nearby parts of the midbrain. Neary and Northcutt (1983) described the bullfrog (*Rana catesbeiana*) diencephalon based on normal histological material, whereas Puelles et al. (1996) proposed a segmental framework based on acetylcholinesterase histochemistry in the frog *Rana perezi*, which was later confirmed by additional markers in *R. perezi* and *Xenopus laevis* by Milan and Puelles (2000). For the thalamus, divisions based on Neary and Northcutt (1983) were used here, as they were applied in the fire-bellied toad by Roth et al. (2003) and Laberge et al. (2008). Nomenclature and regional boundaries in the ventral diencephalon and midbrain are more contentious. In these regions, the neuroanatomical frameworks of Neary and Northcutt and Puelles et al. differ in two major respects: (1) The posterior tuberculum of Neary and Northcutt is a large structure with dorsal and ventral parts, the latter of which includes the mammillary nucleus (Ma) that Puelles et al. placed in the dorsal hypothalamus. (2) Puelles et al. divide the posterior tubercle into the nucleus of the tuberculum posterior (TP) proper and retromammillary nucleus (RM), which is inserted below the TP. RM is in place of the rostral extension of the nucleus of the periventricular organ (NPv) of Neary and Northcutt.

The present study is not an attempt to locate all the subdivisions of the ventral diencephalon mentioned above, as no clear divisions could be detected within the fire-bellied toad posterior tubercle and ventral tegmentum. The framework used for presentation of the data is a simplified version based on Puelles et al. (1996) that considers posterior tubercle and ventral tegmentum as undivided but distinct units. Since the latter two regions appeared to form a rostrocaudal continuum without an intervening structure in our experiments, we did not consider that a nucleus of the medial longitudinal fasciculus (NMLF) was inserted between the posterior tubercle and tegmentum rostrocaudally, as Neary and Northcutt (1983) and Puelles et al. (1996) did. Further work might of course uncover subdivision details within these regions.

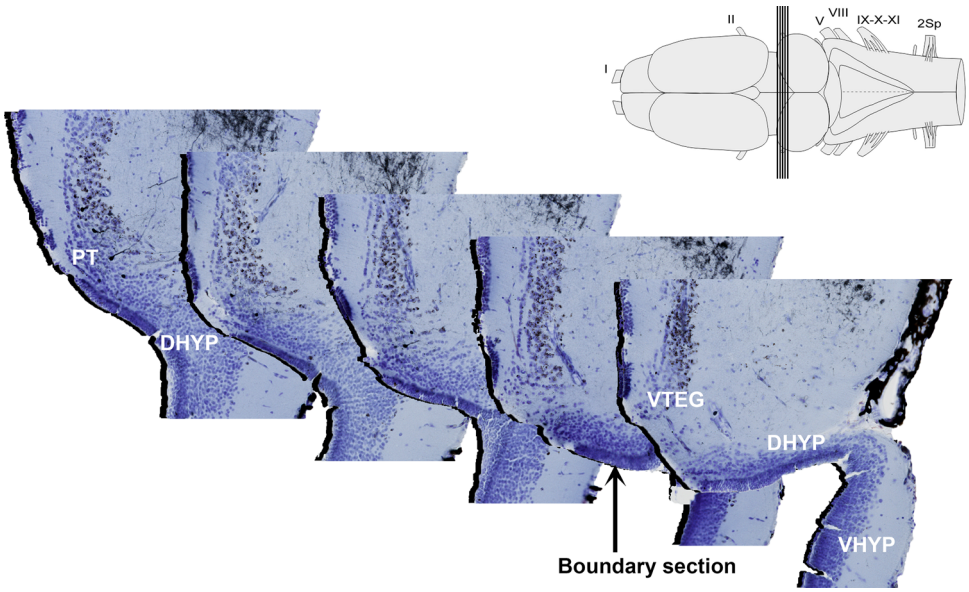

Fig. 1 Boundary between posterior tubercle and ventral tegmentum in the fire-bellied toad. The micrographs show five consecutive 50 μm transverse brain sections that include part of the ventral diencephalon/midbrain and hypothalamus. The most rostral section is to the *left* and rostrocaudal levels of section are indicated on the dorsal schematic view of the brain in the *top right corner*. The boundary section at the limit between the posterior tubercle and ventral tegmentum is shown with an *arrow*. This was taken from an experiment where biocytin was applied to the dorsal striatopallidal system on the *right* half of the brain. Intense biocytin labelling (*black*) of descending and backfilled axons can be seen in the *white matter* on the *top right* of micrographs, while backfilled somata of neurons and some of their dendrites can be seen in the posterior tubercle. *Cresyl violet* counterstain and *dark* pigments in the posterior tubercle and tegmentum outline the *grey matter*. *I-XI* cranial nerves, *2Sp* second spinal nerve, *DHYP* dorsal hypothalamus, *PT* posterior tubercle, *VHYP* ventral hypothalamus, *VTEG* ventral tegmentum

A crucial neuroanatomical landmark for the present study is the boundary between the posterior tubercle and the most rostral part of the ventral tegmentum, which could separate the regions of origin of possibly distinct tubercular and midbrain ascending pathways to the striatopallidal systems. This boundary is defined here as the rostrocaudal level where the periventricular grey matter of the hypothalamus and diencephalon becomes separate in the transverse plane of section (Fig. 1). In sections rostral to the boundary, the periventricular grey matter of anterior hypothalamus and diencephalon is continuous, whereas caudal to the boundary it separates into dorsal hypothalamus ventrally and ventral tegmentum dorsally. This demarcating line could always be attributed with confidence to one 50 μm-thick transverse brain section. Further, differences in neuron soma size and dendritic tree orientation (see below) allowed attribution of a tubercular or tegmental nature to neurons labelled in the boundary section.

Results

Application sites

Table 1 lists the biocytin applications that were considered restricted to one of the dorsal or ventral striatopallidal systems (DSP and VSP, respectively) and provides details of rostrocaudal location as well as leakage to neighbouring brain regions. When it occurred, this leakage to neighbouring regions was very small. Attribution of restricted applications was facilitated by a clear separation of

Table 1 Location of biocytin application sites restricted to a striatopallidal system

Region	Applications[a]	
	DSP ($n = 9$)	VSP ($n = 8$)
Rostral	3[b]	2
Intermediate	5[c]	4[d]
Caudal	0	1[e]
Entire	0	1[f]
Intermediate + caudal	1	0

[a] Three of the DSP and VSP applications were made on the left side of the brain, while the remaining applications were on the right side

[b] One application site included part of the caudal main olfactory bulb

[c] Three application sites included only the ventral part of the DSP

[d] Three application sites included a small part of the ventromedial septum, one additionally included a small part of the ventral DSP

[e] This application site also included some of the amygdala and caudal DSP region

[f] This application site also included a small part of the ventral DSP

DSP dorsal striatopallidal system, *VSP* ventral striatopallidal system

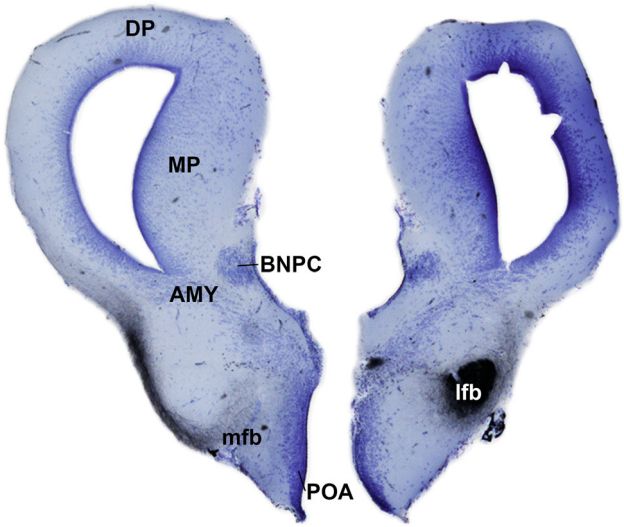

Fig. 2 Differential labelling of forebrain bundles following biocytin applications restricted to the dorsal and ventral striatopallidal systems. The micrographs show 50 µm transverse brain sections taken at the level of the caudal amygdala in the same toad, where the brain was split longitudinally and biocytin was applied at different sites. Applications were restricted to the ventral striatopallidal system on the *left* half of the brain and to the dorsal striatopallidal system on the *right* half. Biocytin labelling of bundles (*black*) can be distinguished from the *cresyl violet* counterstain by its distinct appearance and stronger intensity. *AMY* amygdala region, *BNPC* bed nucleus of the pallial commissure, *DP* dorsal pallium, *lfb* lateral forebrain bundle, *mfb* medial forebrain bundle, *MP* medial pallium

dendritic territories at the DSP-VSP border; dendrites of DSP neurons do not cross into the VSP territory, and vice versa. Two methods were used to confirm that the small leakage of biocytin to regions outside of the striatopallidal systems would not impact the results. First, axon labelling had to be restricted to the lateral (DSP applications) or medial (VSP applications) forebrain bundle (Fig. 2). Second, retrograde labelling had to strongly favour the anterior thalamic nucleus for VSP applications and the lateral and central thalamic nuclei for DSP applications, as expected from previous work in the fire-bellied toad (Laberge et al. 2008). Figures 3 (DSP) and 4 (VSP) each show an example of restricted biocytin applications and the resulting location of backfilled neurons in regions of interest. Additional biocytin applications that were not restricted to a single striatopallidal system ($n = 15$) were used for the correlation analysis presented below.

Retrograde labelling in the thalamus

After restricted biocytin applications to the DSP, the great majority of neurons backfilled in the thalamus were found in the ipsilateral lateral and central thalamic nuclei (Fig. 3). A few neurons were sometimes seen at the lateral edge of the anterior thalamic nucleus. The opposite was true of applications restricted to the VSP, where backfilling strongly favoured the anterior thalamic nucleus in the rostral diencephalon (Fig. 4). The small amount of backfilling outside of expected thalamic regions was also seen in biocytin applications that were perfectly restricted to a striatopallidal system. It could mean that there is a mixing of somata with distinct ascending projections to the telencephalon near nuclear boundaries in the dorsal thalamus. Figure 5a shows the averaged rostrocaudal distribution of backfilled neurons on 50 µm-thick transverse brain sections following biocytin applications restricted to the striatopallidal systems. The distribution of neurons in the dorsal thalamus showed no clear rostrocaudal topographical relationship despite the fact that different application sites involved different nuclei. Backfilling that resulted from applications to the DSP and VSP began at the same rostral level in the lateral and anterior thalamic nuclei, and backfilled neurons extended almost as far caudally in the central and anterior thalamic nuclei in both cases. This resulted from the presence of neurons in the caudal part of the anterior thalamic nucleus; a thin layer sandwiched between the central and ventromedial thalamic nuclei. The presence of a greater number of backfilled neurons following applications to the DSP was most likely the result of larger application sites compared to the VSP, which was more difficult to target with biocytin applications because of its smaller size.

Retrograde labelling in the ventral diencephalon/midbrain

The rostrocaudal distribution pattern of backfilled neurons in the posterior tubercle and ventral tegmentum differed markedly from what was seen in the dorsal thalamus (Fig. 5b). DSP applications resulted in a peak of backfilled neurons in the caudal part of the posterior tubercle (the transition between the posterior tubercle and the tegmentum is located between sections #22 and 23 in Fig. 5), while most of the backfilled neurons were located in the ventral tegmentum following VSP applications. Labelling heavily favoured the ipsilateral side in preparations where it could be observed. Labelled neurons in the posterior tubercle had larger somata with thicker proximal dendrites that fanned out laterally, whereas tegmentum neurons had smaller somata and dendrites oriented ventrally. These features were helpful to distinguish labelled tubercular and tegmental neurons in the boundary section between ventral diencephalon and midbrain. In all cases, backfilled neurons were present in both the posterior tubercle and the ventral tegmentum. The proportion of backfilled neurons in the posterior tubercle was calculated for each toad by dividing the number counted in the posterior tubercle by the total number of backfilled neurons in the posterior tubercle and

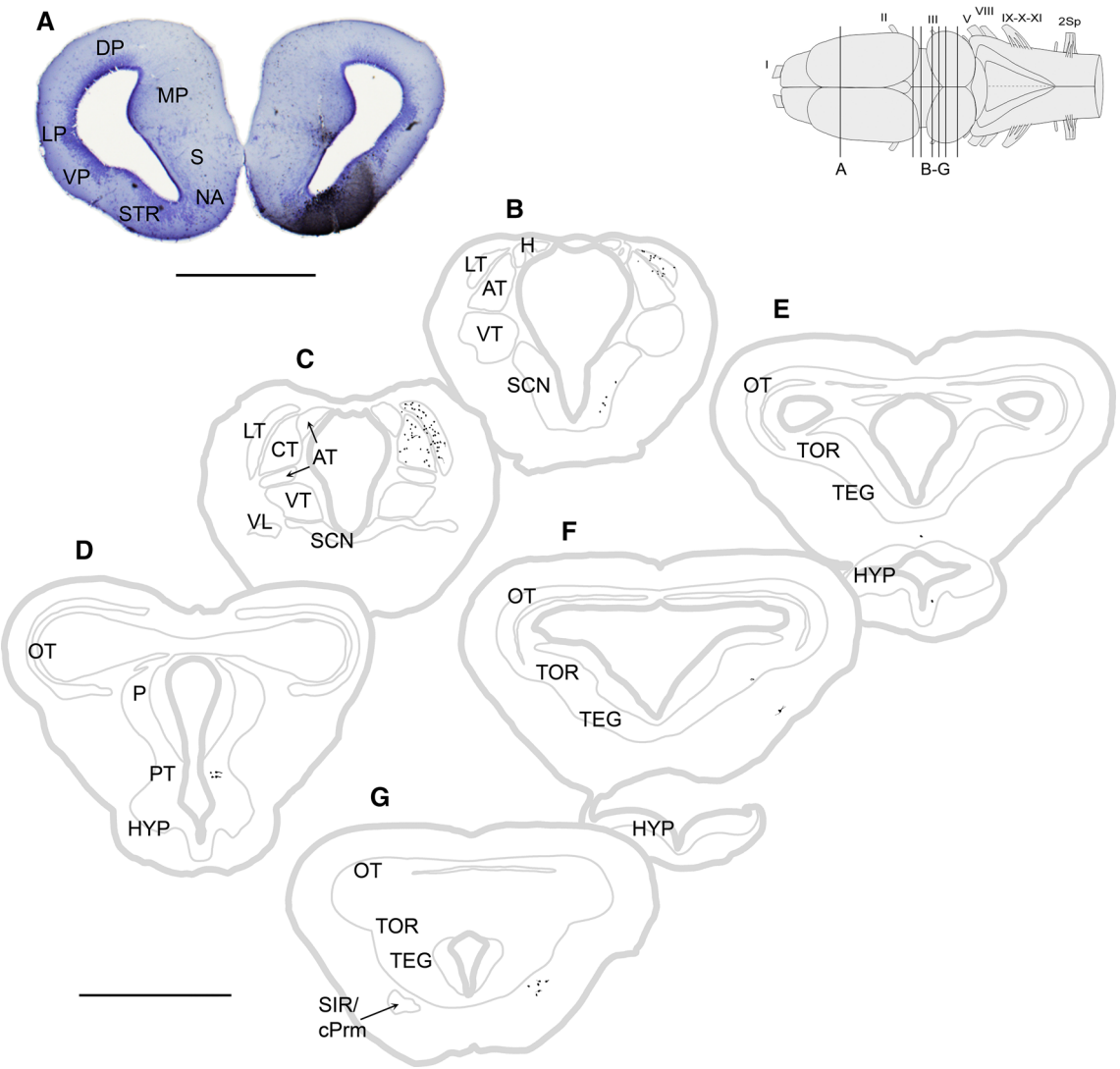

Fig. 3 Example of a biocytin application restricted to the dorsal striatopallidal system and the resulting backfilling of neurons in regions of interest. **a** Photomicrograph showing the application site in the dorsal striatum (*black*) on the *right* side of the brain. **b–g** Camera lucida drawings of brain outlines (*grey*) and cell bodies of backfilled neurons (*black*). Levels of section are illustrated on the schematic dorsal view of the brain on the *top right* and brain structures are labelled on the *left side* of sections. *Scale bars* are 1 mm. *I-XI* cranial nerves, *2Sp* second spinal nerve, *AT* anterior thalamic nucleus, *CT* central thalamic nucleus, *DP* dorsal pallium, *H* habenula, *HYP* hypothalamus, *LP* lateral pallium, *LT* lateral thalamic nucleus, *MP* medial pallium, *NA* nucleus accumbens, *OT* optic tectum, *P* posterior thalamic nucleus, *PT* posterior tubercle, *S* septum, *SCN* suprachiasmatic nucleus, *SIR/cPrm* superficial isthmal reticular nucleus/caudal profundus, *STR* dorsal striatum, *TEG* tegmentum, *TOR* torus semicircularis, *VL* ventrolateral thalamic nucleus, *VP* ventral pallium, *VT* ventral thalamic nucleus

ventral tegmentum. Backfilling in DSP applications that were successful on both sides of split brain preparations ($n = 3$) was averaged to avoid pseudo replication. A t test showed that the proportion of backfilled neurons in the posterior tubercle was significantly higher in DSP applications ($M = 0.85$, 95 % CI [0.76, 0.94]) than in VSP applications ($M = 0.25$, 95 % CI [0.12, 0.37]), $t(15) = 9.35$, $p < 0.0001$.

We used 15 biocytin applications that were not restricted to a single striatopallidal system to further support the above finding of distinct ascending thalamostriatal and mesostriatal pathways. Four biocytin applications with less than 10 backfilled neurons were rejected from this analysis to avoid possible bias due to small samples. We reasoned that applications targeting mostly one striatopallidal system should show greater backfilling in the expected regions, as in the restricted applications described above, while applications involving both striatopallidal systems equally should not reveal a topographical relationship. A correlation analysis of the proportion of backfilled neurons in the lateral/central thalamic nuclei and posterior tubercle showed that these two variables were strongly correlated

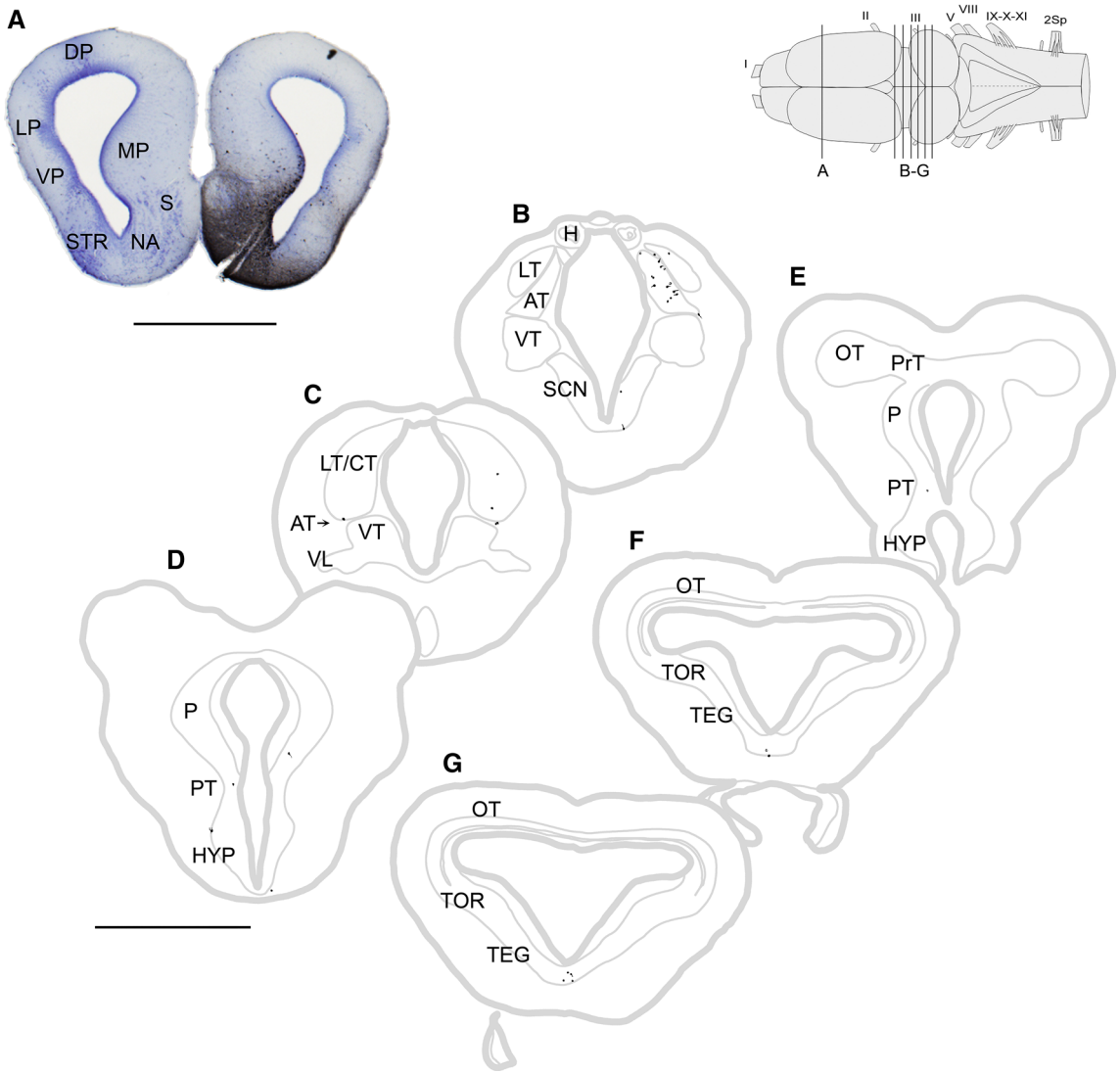

Fig. 4 Example of a biocytin application restricted to the ventral striatopallidal system and the resulting backfilling of neurons in regions of interest. **a** Photomicrograph showing the application site in the nucleus accumbens (*black* and surface lesion) on the *right side* of the brain. **b–g** Camera lucida drawings of brain outlines (*grey*) and cell bodies of backfilled neurons (*black*). Levels of section are illustrated on the schematic dorsal view of the brain on the *top right* and brain structures are labelled on the *left side* of sections. *Scale bars* are 1 mm. *I-XI* cranial nerves, *2Sp* second spinal nerve, *AT* anterior thalamic nucleus, *CT* central thalamic nucleus, *DP* dorsal pallium, *H* habenula, *HYP* hypothalamus, *LP* lateral pallium, *LT* lateral thalamic nucleus, *MP* medial pallium, *NA* nucleus accumbens, *OT* optic tectum, *P* posterior thalamic nucleus, *PrT* pretectum, *PT* posterior tubercle, *S* septum, *SCN* suprachiasmatic nucleus, *STR* dorsal striatum, *TEG* tegmentum, *TOR* torus semicircularis, *VL* ventrolateral thalamic nucleus, *VP* ventral pallium, *VT* ventral thalamic nucleus

[Pearson $r(13) = 0.70$, $p = 0.0035$, Fig. 6]. Thus, the topographical relationships seen with restricted biocytin applications could also be seen when one striatopallidal system was preferentially, but not exclusively, labelled.

Retrograde labelling in other brain regions

Table 2 summarises the retrograde tracing results outside of the thalamus and ventral diencephalon/midbrain for the restricted DSP and VSP biocytin applications. In almost all cases, mitral cells in the main olfactory bulb were labelled. The strongest such labelling was seen after VSP applications because the medial olfactory tract runs at the surface of this region in *B. orientalis* (Roth et al. 2004). Strong retrograde labelling in the rostral part of a striatopallidal system was seen following caudal application to the same striatopallidal system, as was expected (Endepols et al. 2004a; Roth et al. 2004). Application to the VSP also sometimes produced some backfilling in the DSP, and vice versa, suggesting the presence of connections between the

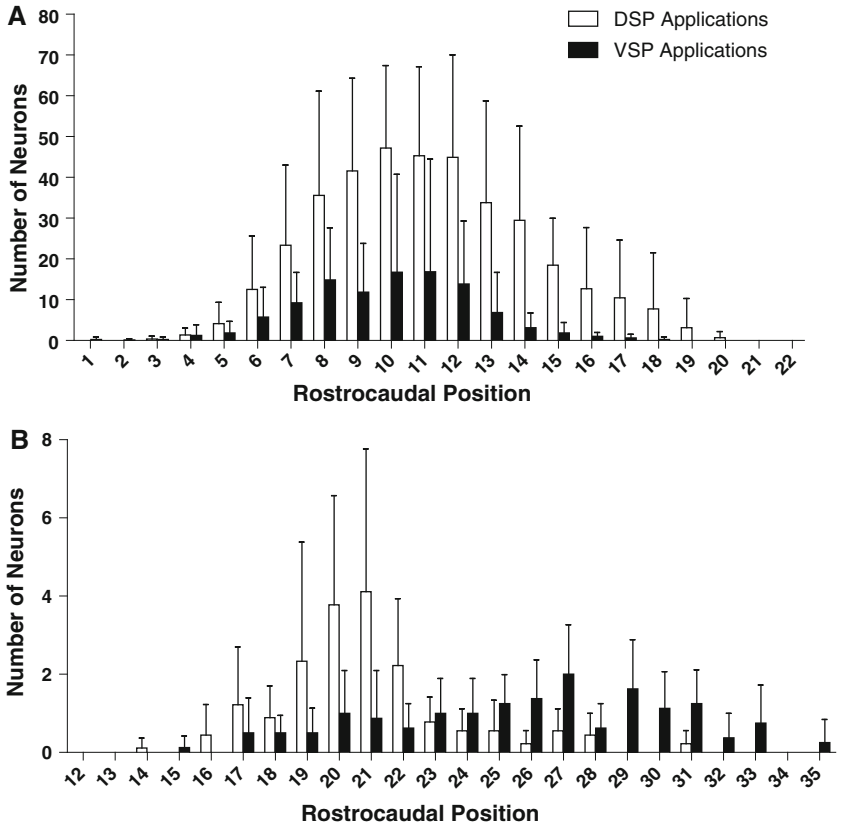

Fig. 5 Rostrocaudal distribution of retrograde labelling in the dorsal thalamus and ventral diencephalon/midbrain after restricted applications of biocytin to the striatopallidal systems of the fire-bellied toad. **a** Retrograde labelling in the dorsal thalamus. *White bars* show the number of backfilled neurons per brain section in the lateral and central thalamic nuclei following applications to the dorsal striatopallidal system. *Black bars* show the number of backfilled neurons in the anterior thalamic nucleus following applications to the ventral striatopallidal system. **b** Retrograde labelling in the posterior tubercle and ventral tegmentum. *White bars* show the number of backfilled neurons per brain section following applications to the dorsal striatopallidal system, while *black bars* shows it for applications to the ventral striatopallidal system. Data are presented as mean +95 % CI. On the horizontal axis of both figure panels, each 50 μm brain section was numbered in reference to the boundary between the posterior tubercle and ventral tegmentum, which is located between sections #22 and 23. Backfilled neurons in the caudal dorsal thalamus were found above those labelled in the posterior tubercle within the same transverse brain sections

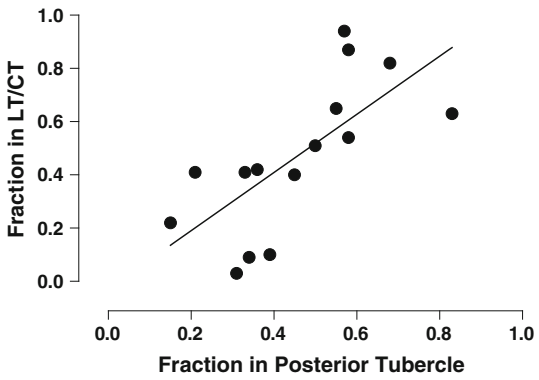

Fig. 6 Correlation of retrograde labelling in the dorsal thalamus and posterior tubercle in cases where biocytin applications covered parts of both striatopallidal systems. The fractions in lateral/central thalamic nuclei (LT/CT) and posterior tubercle were obtained by dividing by the total number of backfilled neurons in the thalamus or ventral diencephalon/midbrain, respectively. The *curve* was fitted using linear regression

two striatopallidal systems. The septum is a major source of input to the striatopallidal systems, as strong backfilling was seen in the lateral septum in almost all applications. However, the medial septum selectively targets the VSP.

Retrograde labelling was limited to three pallial regions: (1) The ventral pallium (formerly striatopallial transition area), which sends fibres superficially above the DSP on their way to the VSP. The location of these fibres made them unavoidable when targeting the DSP using the present methods, thus backfilling of the ventral pallium after DSP applications is not proof of a connection between these two regions. (2) As reported by Marín et al. (1997a), neurons in the medial pallium were backfilled following applications to the striatopallidal systems. The rostral part of the medial pallium was usually involved, but applications to the VSP sometimes additionally involved the caudal parts of the medial pallium. (3) As described in Roth et al. (2007), the most rostral part of the pallium sends

Table 2 Intensity of retrograde labelling outside of the ventral midbrain and thalamus following biocytin applications to the striatopallidal systems in *Bombina orientalis*

Region	Application site	
	DSP ($n = 9$)	VSP ($n = 8$)
MOB	+++ (1), + (6), +/− (1), − (1)	+++ (6), + (2)
Rostral pallium	+ (3), − (6)	+/− (2), − (6)
Dorsal striatum (rDSP)	+++ (6), + (2)[a]	+ (2), +/− (3), − (3)
Nucleus accumbens	+++ (2), + (5), +/− (1), − (1)	+++ (5)[a]
Lateral septum	+++ (9)	+++ (6), + (1), − (1)
Medial septum	+/− (2), − (7)	+++ (4), + (4)
Medial pallium	+++ (1), + (5), − (3)	+++ (2), + (4), +/− (1), −(1)
Ventral pallium	+++ (3), + (4), +/− (2)	+ (6), +/− (2)
Amygdala	+ (5), +/− (2), − (2)	+++ (2), + (5), +/− (1)
Preoptic area	+ (2), +/− (4), − (3)	+++ (2), + (3), +/− (3)
BM	+ (3), +/− (3), − (3)	+ (2), +/− (1), − (5)
SCN	+ (4), +/− (2), − (3)	+/− (1), − (7)
Dorsal hypothalamus	+ (2), +/− (1), − (6)	+ (1), +/− (5), − (2)
Ventral hypothalamus	+/− (3), − (6)	+++ (2), +/− (2), − (3), missing (1)
SIR/cPrm	+++ (2), + (5), +/− (1), − (1)	−(8)
Locus coeruleus	+ (1), +/− (1), − (7)	+/− (2), − (6)
Parabrachial nucleus	+ (3), +/− (3), − (3)	+ (2), +/− (4), − (2)
Raphe median	−(4), missing (5)	+/− (4), − (3), missing (1)

Numbers in parentheses indicate the number of experiments where retrograde labelling of that intensity was observed in said region. For three toads where DSP applications were successful in both halves of the split brains, only the half with the most abundant retrograde labelling is included

Backfilling intensity: none (−), weak (+/−), moderate (+), strong (+++)

[a] Some application sites included these regions

BM bed nucleus of the stria medullaris of Neary and Northcutt (1983), *DSP* dorsal striatopallidal system, *MOB* main olfactory bulb, *SCN* suprachiasmatic nucleus, *SIR/cPrm* superficial isthmal reticular nucleus/caudal profundus of Maier et al. (2010), *VSP* ventral striatopallidal system

afferents to both striatopallidal systems. This was seen by moderate or weak backfilling in about half of biocytin applications.

In the hypothalamus, neurons were often seen in the suprachiasmatic nucleus after DSP, but not VSP applications. Results in other parts of the hypothalamus did not show clear trends. In more caudal brain regions, the superficial isthmal reticular nucleus/caudal profundus (SIR/cPrm) of Maier et al. (2010) was retrogradely labelled only with DSP applications (Fig. 3g), while the locus coeruleus and parabrachial nucleus were sometimes labelled after applications to either DSP or VSP.

Applications to the ventral diencephalon/midbrain

Anterograde labelling following biocytin applications to the posterior tubercle or ventral tegmentum confirmed the validity of our retrograde labelling observations (Table 3). The presence of only moderate to weak projections to the striatopallidal systems is in line with the observations made using retrograde labelling and suggests that only a limited number of neurons in the posterior tubercle and ventral tegmentum send ascending projections to the striatopallidal systems.

Discussion

Applications of the sensitive neuronal tracer biocytin showed that the striatopallidal systems in the fire-bellied toad receive distinct thalamic input potentially under influence of ascending projections from either the posterior tubercle or ventral tegmentum. Therefore, there are two mesostriatal pathways in *B. orientalis*: a dorsal pathway between posterior tubercle and DSP and a ventral pathway between ventral tegmentum and VSP. A small overlap in input from the posterior tubercle and tegmentum might be due to mixing between cells of origin of the two mesostriatal pathways along the rostrocaudal axis of the ventral diencephalon/midbrain or the presence of neurons that send axons to both striatopallidal systems, but this will require further investigation.

Table 3 Summary of anterograde projections following biocytin applications to ventral diencephalon/midbrain sites in *Bombina orientalis*

Axonal projection sites[a]	Biocytin application sites				
	VTEG ($n = 2$)	PT ($n = 2$)	PT + rVTEG[c] ($n = 2$)	PT + DHYP ($n = 3$)	PT + SCN ($n = 1$)
Nucleus accumbens (rVSP)	+/−	−, +	−, +++	+/−, −, −	+
Dorsal striatum (rDSP)		+	−, +++	−, +/−, −	+/−
Medial pallium			−, +++[d]		
Lateral septum			−, +	+, −, −	
Medial septum		−, +/−	−, +	+, −, +	+
Preoptic area	+	+	+, ?	+	+
Medial amygdala	?, −[b]	−, ?	−, +	?, −, +	?
Thalamic eminence		−, +	−, +	+++, −, −	+
Anterior thalamic nucleus	+, −	+	−, +	+, −, −	+
Medial thalamic neuropil	+, −	+, −	−, ?	+++, −, −	+
Ventral thalamus		−, +++	−, +	+++, +, +++	+
Lateral hypothalamus	?, −	+, −	?, −		
Ventral tegmentum		+	−, +	+++, −, +	+
Dorsal tegmentum		+	−, +		
Optic tectum		−, +	−, +	+, +, −	
Torus semicircularis		+	−, +	+, −, −	
Parabrachial nucleus region			−, +		
Medulla oblongata	+	+	+	+	+

[a] Projection intensity: none (−), weak (+/−), moderate (+), strong (+++), ? (difficult to assess). Blank cells indicate that no experiment resulted in projections to that site

[b] When experiments involving the same application site produced different results, each result is separated by a comma

[c] Second biocytin application was a large one

[d] Only rostroventral part of medial pallium

DHYP dorsal hypothalamus, *DSP* dorsal striatopallidum, *PT* posterior tubercle, *r* rostral, *SCN* suprachiasmatic nucleus, *VSP* ventral striatopallidum, *VTEG* ventral tegmentum

Evolution of striatopallidal systems

The bilateral basal ganglia are sometimes considered as undivided units made up of a core neural substrate differentiated only by topographically distinct patterns of afferents (Voorn et al. 2004). An alternative view considers two major divisions of the basal ganglia: the dorsal and ventral striatopallidal systems (Heimer et al. 1982; Marín et al. 1998b). Marín et al. (1998b) have proposed that the pattern of basal ganglia organised into dorsal and ventral systems is a conserved feature of tetrapod vertebrates. This division into adjacent but distinct dorsal and ventral striatopallidal systems is supported by the pattern of dendritic labelling in *B. orientalis* that followed restricted biocytin applications. Namely, despite continuous cellular layers in the grey matter of the DSP and VSP, a sharp boundary between dendritic compartments of each striatopallidal system was observed in the ventromedial telencephalon.

The presence of markers of the striatopallidal system was demonstrated in the cerebral hemispheres of fishes (Reiner et al. 1998; Wullimann and Mueller 2004). Distinct striatal and pallidal parts can be recognised in basal fishes (dogfish: Northcutt et al. 1988; lamprey: Stephenson-Jones et al. 2011), but they appear intermingled into a single nucleus in actinopterygians (Reiner and Northcutt 1992; Wullimann and Mueller 2004). Interestingly, a recent study proposed that distinct dorsal and ventral striatopallidal systems are present in the lungfish cerebral hemispheres (Northcutt 2009). This situation suggests that two major types of evolutionary change took place in the striatopallidal systems during vertebrate phylogeny: (1) duplication of the single striatopallidal system inherited from basal fishes in sarcopterygians followed by specialisation into dorsal and ventral divisions, and (2) the opposite trend where the distinct striatal and pallidal parts of the striatopallidal system inherited from basal fishes were merged into a single structure in the lineage leading to actinopterygians.

Evolution of mesostriatal pathways

In mammals, functional divisions of the mesostriatal system are often recognised (Wise 2004; Ikemoto 2007). Particularly, dopaminergic innervation of the dorsal

striatum originating in the substantia nigra pars compacta (SNc) is known as the nigrostriatal pathway, whereas the mesolimbic system refers to a projection to the ventral striatum (nucleus accumbens and olfactory tubercle) that originates from neurons in the ventral tegmental area (VTA). A subset of VTA neurons located medially additionally defines the mesocortical system by sending axon collaterals to cortical regions in addition to a projection to the nucleus accumbens (Lindvall et al. 1974; Lammel et al. 2008). A connection between the mesencephalon and the cortex is seen in reptiles, but its neurochemistry is not determined (Bruce and Butler 1984). Collaterals of mesensephalic neurons to the pallium appear to be absent in *B. orientalis* and dopamine fibres are also absent from pallial regions in the anurans that were studied (González and Smeets 1991; González et al. 1993). Thus, the mesocortical projection might be new in amniotes. Interestingly, a pathway functionally equivalent to the mesocortical projection system has been found in the avian brain, but it projects to a distinct part of the cerebral cortex and may not be homologous to its equivalent in mammals (Güntürkün 2005).

The structures giving rise to the mammalian mesostriatal pathways are believed to form a continuum that spans the ventral diencephalon, midbrain and isthmus in the rostrocaudal axis of the brain (Marín et al. 1998b; Reiner et al. 1998). Thus, the situation in amphibians, where both diencephalic tubercular and midbrain tegmental regions give rise to mesostriatal pathways, could be largely comparable to the mammalian situation (Smeets and González 2000; present results). Actinopterygian fish are also reported to display ascending projections to the telencephalic subpallium, where a putative striatum could be located; however, in most species studied, the cells at the origin of this pathway are found only in the diencephalon (Reiner et al. 1998; Rink and Wullimann 2001). The present results show that the tubercular and tegmental populations have distinct, albeit somewhat overlapping, striatopallidal targets. Such an overlap is also a feature of the SN/VTA of mammals, where these regions send striatal projections organised in general, but not exclusive, mediolateral topography (Fallon and Moore 1978; Ikemoto 2007). The present results confirm the previous suggestion of Marín et al. (1997b) according to which the topographical organisation of cells at the origin of mesostriatal pathways in amphibians is along the rostrocaudal axis of the brain, without the mediolateral gradient seen in most amniotes.

Function of mesostriatal pathways

Functional coupling between posterior tubercle and dorsal pallidum has been suggested based on a positive correlation between expression of the immediate-early gene *egr-1* in the dorsal pallidum and the size of the tyrosine hydroxylase-expressing tubercular population in Túngara frogs (*Physalaemus pustulosus*) exposed to advertisement calls (Hoke et al. 2007). The presence of putative dopaminergic neurons in this pathway could suggest a role in reward processing (Wise 2004). Indeed, a subset of neurons forming the mesostriatal pathways in amphibians has been shown to express dopamine (González and Smeets 1991; González et al. 1993; Parish et al. 2007; O'Connell et al. 2010; Yamamoto and Vernier 2011). The dual mesostriatal pathways present in amphibians could display a division of tasks in motivational (ventral) and motor control (dorsal), as suggested in mammals. Indeed, lesion of diencephalic/midbrain dopaminergic neurons in amphibians leads to important motor deficits reminiscent of Parkinson's disease (Endepols et al. 2004b; Parish et al. 2007). However, it should be noted that a clear distinction between motivational and motor roles for the two mesostriatal pathways is not supported by recent work showing reward learning in the dorsal striatum (Yin et al. 2008).

Work in mammals has shown that neurons projecting to the nucleus accumbens can express neurotransmitters other than dopamine. Mesocortical neurons can express glutamate only or co-express glutamate and dopamine (Yamaguchi et al. 2011), while mesolimbic neurons expressing the inhibitory neurotransmitter GABA have been shown to control reward-related behaviours (Van Bockstaele and Pickel 1995; van Zessen et al. 2012). These neurotransmitters could coordinate multiple aspects engaged when learning about rewards and these functions could be shared among amphibians and amniotes. Interestingly, dopamine itself has been proposed to play multiple roles in appetitive learning, such as promoting increased attention towards alerting events and encoding motivational value and salience of goals that are sought (Alcaro et al. 2007; Bromberg-Martin et al. 2010). More research on the respective roles of the different neurotransmitter systems ascending to the striatopallidal systems is clearly needed. Amphibians could provide a useful comparative model to investigate the function of mesostriatal neurons that express neurotransmitters other than dopamine because their dopaminergic projections to the striatopallidal systems are much sparser than in amniotes (Reiner et al. 1998), and thus the importance of dopamine in mediating the effects of reward might be comparatively less than in amniotes.

Subthalamic nucleus candidate in amphibians

Recently, Maier et al. (2010) sought evidence for an indirect basal ganglia pathway in *B. orientalis* and proposed different candidate structures for the subthalamic nucleus.

The candidate regions displayed adequate neurochemistry as well as afferents from the dorsal pallidum and efferents to the region of the tegmentum considered as substantia nigra pars reticulata (SNr) by the authors. However, all candidates lacked one characteristic of the mammalian subthalamic nucleus; a projection back to the pallidum. We propose that the SIR/cPrm should be considered a potential candidate for the subthalamic nucleus due to its projection back to the DSP, its close location to the amphibian SNr, and the inhibitory input it receives from what is likely the dorsal pallidum (Wu and Wang 2007). The small sample size and different method of neurotracer application used by Maier et al. (2010) might have prevented observation of the connection from the SIR/cPrm to the pallidum in their study. The close proximity of SNr and SIR/cPrm within the tegmentum should make demonstration of an association between these two regions difficult using anatomical methods. Thus, investigation of the physiology of the SIR/cPrm might be required to evaluate if it is a plausible candidate for the subthalamic nucleus in amphibians.

Conclusion

The presence of dual striatopallidal systems and mesostriatal pathways in amphibians and amniotes suggests that ancestral tetrapods already had both dorsal and ventral systems. More work is needed in fish to find out if this condition was inherited from fish ancestors or was new to tetrapods. Investigation of the mesostriatal pathways in lungfish would be of great interest in that respect (see Northcutt 2011 on the need for more study in lungfishes). The present results support functional equivalence of the amphibian posterior tubercle and mammalian substantia nigra, while the mammalian ventral tegmental area appears equivalent to the amphibian ventral tegmentum.

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