Evidence for a cortical–basal ganglia projection pathway in female zebra finches

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The anterior forebrain pathway in songbirds is a specialization of the avian basal ganglia pathway and is prominent in males that sing, but seem to be absent or incomplete in females that do not sing. We studied the connectivity in females in the in vitro slice preparation by applying the tracer Fluoro Ruby, biotinylated dextran amine, and cholera toxin B. We identified (1) retrograde labeled neurons in the lateral magnocellular nucleus of the anterior nidopallium (LMAN) projecting to the medial striatum (MSt), and (2) we identified fibers in the MSt labeled by anterograde transport after tracer injection into LMAN. Our data clearly demonstrate the existence of a cortico-basal ganglia pathway in female birds. NeuroReport 16:21–24 © 2005 Lippincott Williams & Wilkins.

Key words: Anterior forebrain pathway; BDA; CtB; Fluoro-Ruby; In vitro slice preparation; Song system nuclei; Zebra finch

INTRODUCTION

Songbirds have evolved specialized brain nuclei that are devoted to song learning and perception and to generate song. In songbird species such as the zebra finch, where only males sing, and females do not, song system nuclei are much smaller in female birds [1,2]. However, there seems to be at least one exception to this rule and that is the cortical lateral magnocellular nucleus of the anterior nidopallium (LMAN), which in females is as large as in males [3] (for nomenclature see [4]). In males, LMAN is part of the anterior forebrain pathway (AFP) and this pathway has been shown to be essential for song-learning processes in juvenile birds and for maintenance of song in adulthood, but it is not required for song production [5–7]. The AFP consists of three nuclei connected in a loop: LMAN, Area X in the medial striatum (MSt) of the basal ganglia, and the medial portion of the dorsolateral thalamic nucleus (DLM). The AFP in songbirds is considered to be a specialized projection system resembling the cortico-basal ganglia-thalamocortical pathway in mammals [8,9].

Much of our knowledge about this pathway in songbirds comes from studies on males; little is known about whether this pathway exists in female birds that do not sing. Data suggest that nonsinging females make use of song system nuclei for song perception without being able to produce song, and LMAN in the AFP might play an important role in this process [10–12]. However, a well-defined region of Area X in the MSt is absent from female zebra finches and, therefore, a projection from LMAN to MSt may be lacking. To give an answer to this question, we have investigated the connectivity between cortical LMAN and the avian basal ganglia region MSt in the in vitro slice preparation using a variety of neuronal tracers. Our data clearly demonstrate that neurons in female LMAN project to MSt in the basal ganglia.

MATERIALS AND METHODS

Animals and slice preparations: All experiments were conducted in accordance with the national laws for the use of animals in research and approved by the local ethical committee. Brain slices were prepared as described previously [13]. Briefly, 14 adult females and 18 adult males were anesthetized, decapitated, and brains were rapidly removed and placed in ice-cold artificial oxygenated cerebrospinal fluid (ACSF). The two hemispheres were separated and whole-brain sagittal sections (400μm) were cut using a vibrpisher. After removing the tip of the lateral pole, 4–5 slices were transferred into an interface chamber, and after a recovery period of 1–1.5 h, individual slices were injected with selected tracers.

Neuronal tracer and injection sites: Neuronal tracers were bidirectionally transported dextran amines (mol. wt 10000; from Molecular Probes Europe, Leiden, NL) and the retrograde tracer cholera toxin subunit B (CtB, Sigma, Deisenhofen, Germany). Glass micropipettes (tip diameter 20μm) were filled with either 25% fluorescent tetra-methylrhodamine dextran amine (Fluoro-Ruby, FR), 10% non-fluorescent tracer biotinylated dextran amines (BDA), or 1% CtB and mounted to a mechanical pressure device. The tracer was pressure injected into MSt in females and Area X in males, and into LMAN in both sexes, under optical control using a binocular microscope. The smallest applied volume with successful tracer application was 4.6 nl. We used a total of 10 females for tracer application into MSt (6 for FR, 3 for BDA, 1 female for CtB) and a total of 14 males (7 for FR, 4 for CtB, 3 for BDA). Injections into LMAN were carried out using FR in 4 males and 4 females. After dye application the slices remained in the chamber for 12–14 h.
Histological processing: The slices were removed from the chamber, fixed in 4% paraformaldehyde and cryoprotected with 30% sucrose at 4°C. Serial sections (40 μm) were cut on a freezing microtome and either mounted on gelatin-coated glass slides (FR experiments) or stored in phosphate-buffered saline (PBS) for further processing BDA or CtB. Sections mounted on glass slides were air-dried and coveredslipped using non-fluorescent mounting media. For identification of brain regions when using fluorescent tracer, the section before the last cut from one vibratome slice was mounted separately on a glass slide, counterstained with cresyl violet, and coverslipped.

BDA histochemistry: Free-floating sections were rinsed in 0.1 M PB and incubated for 30 min with 0.3% H2O2 in PB to inhibit endogenous peroxidase activity. After an overnight incubation in a standard avidin/biotinylated horseradish peroxidase complex (Vectastain Elite Kit, Camon, Wiesbaden, Germany), sections were washed several times and mounted on glass slides for further processing. Dried sections were developed under visual control using the glucose oxidase-DAB-nickel method [14]. Finally, sections were rinsed in buffer and counterstained with 5% cresyl violet acetate and coverslipped.

CtB immunohistochemistry: For CtB immunohistochemistry we followed the protocol of Hellmann and Güntürkün [15] with minor modifications. Following minimizing endogenous peroxidase activity, sections were incubated overnight at 4°C in the primary antibody (goat anti-Choleraeindog; Camon, Wiesbaden, 1/40000 in 0.12 M PBS with the addition of 1% NaCl, 0.3% Triton-X-100 and 5% normal goat serum). The next day, sections were rinsed and incubated for 60 min at room temperature in the biotinylated secondary antibody (rabbit anti-goat; Vectastain, Vector, Camon (Wiesbaden, Germany); 1/200 in 0.12 M PBS and 1% NaCl and 0.3% Triton-X-100). After several rinsings, sections were incubated for 60 min in avidin–biotin–peroxidase solution (Vectastain Elite Kit, Camon) and further processed as described for BDA. We were able to verify this projection in all of the three different tracers used in this study. A typical injection into MSt in female birds using the tracer BDA is shown in Figure 2. Axonal fibers in MSt have picked up the tracer and, by retrograde transport, a small cohort of neuronal cell bodies and dendrites was backfilled in LMAn (Fig. 2b). Backfilled neurons were found more or less close together rather than being spread all over LMAn. When using the tracer BDA, we successfully labeled neurons in LMAn in two females out of three, and in two males. The location of the injection site in females is comparable to the location of the injection site in males when injecting into Area X, as illustrated by the injection of the tracer CtB into Area X in males (Fig. 3a). The tracer CtB, characterized by exclusively retrograde transport, was successfully applied in one female and two males, resulting in labeled neurons in LMAn. Using the tracer BDA or CtB often results in ‘Golgi-like-stained-neurons’. We identified fine axon collaterals bifurcating close to the cell soma of retrogradely filled neurons within LMAn in both females and males (Figs. 2b and 3b, respectively).

Injections of FR in six females and seven males revealed labeled neurons in LMAn in two females and three males. Typical fluorescent micrographs after MSt injections in female birds demonstrate intense label of the soma, dendrites, dendritic spines, and fibers in LMAn (Fig. 4). In males, FR applications into Area X also yield labeled neurons in LMAn (Fig. 5a,b). Fluorescent micrographs are depicted to demonstrate the intense labeling of neurons in LMAn in males at low (Fig. 5a) and high magnification (Fig. 5b). In general, we found regional intensity of labeled neurons within LMAn, rather than having backfilled neurons homogenously distributed. Injections into the MSt in females or Area X in males, however, did not always result in retrograde labeling of neurons in LMAn, but sometimes revealed a fine network of fibers in LMAn. These fibers probably represent axons from DLM neurons projecting to LMAn in both males and females. Injections into LMAn revealed an axonal network of fibers in MSt in females and in Area X in males that was labeled by anterograde transport of FR (Fig. 6). We did not find any obvious differences between males and females in labeling of the axonal network.

In summary, our tracer injections into the medial striatum (MSt) of the basal ganglia in female zebra finches and into Area X of the MSt in males, using fluorescent and non-fluorescent tracers, indicate a prominent projection from LMAn to the MSt. We found labeled neurons in LMAn after MSt or Area X injections in 5 females and 7 males.

DISCUSSION

In male songbirds, the AFP consisting of song nuclei LMAn, Area X in the MSt and DLM in the thalamus, has been shown to be topographically organized [9]. So far, very little is known about this projection system in female birds. Here we made the first step, demonstrating the existence of a cortico-basal ganglia projection pathway in female zebra finches using fluorescent and non-fluorescent neuronal tracers in an in vitro slice preparation. In addition to this finding, we identified similarities in the cortico-basal ganglia projection pathway in males and females.

Our observations indicate that, in general, backfilled neurons in LMAn in females are localized in small cohorts rather than being distributed homogenously within LMAn. Therefore, the cortico-basal ganglia projection pathway in
females also might be topographically organized, as has been shown for males using in vivo experiments [9]. In addition to a projection from LMAN to MSt in female birds, we also identified bifurcating axon collaterals of labeled LMAN neurons curving upwards to reach Lamina mesopallialis (LaM). In males, it is well known that axon collaterals of LMAN neurons reaching LaM project to the robust nucleus of the arcopallium (RA) linking the AFP to...
the song motor pathway for song production, while the other axon collateral of the same LMAN neuron projects to Area X in the MSt within the AFP [13,16,17]. In females, bifurcating axon collaterals therefore most likely also project to RA, since the LMAN-RA projection pathway is well known, albeit this projection is strongly reduced in adult females [18]. Alternatively, bifurcating axon collaterals in females may remain within LMAN, contributing to the intrinsic circuitry demonstrated by intracellular recordings techniques in the in vitro slice preparation [19]. Henceforth, LMAN-neurons in female zebra finches are similar in their projection pattern to those in males, exhibiting two projection targets: the MSt as demonstrated in the current report and the well-known projection target RA. In addition to these similarities there are other characteristics in common in song nucleus LMAN, despite the observed sex differences in neuronal morphology (for review see [20]). The number of axonal profiles, for example, increases in both males and females during development and remains enhanced in adulthood [21]. It has also been shown that neurons in LMAN receive input from DLM in the thalamus in males and females [19]. Evidence for a connectivity between the basal ganglia and the thalamus in female zebra finches, however, is still lacking and has not yet been verified in literature.

Female zebra finches do not sing, but they do memorize their species-specific song and are able to discriminate songs of greater and lesser complexity [22]. Song nucleus LMAN within the AFP has been shown to play an important role in these processes [11,12]. We suggest that females make use of the same song control pathway in the brain that males do, but future research needs to address questions on how and where in the basal ganglia loop the cognitive ability of perception is localized and mirrored in neuronal coding and neuronal substrate.

**CONCLUSION**

We have demonstrated that song nuclei of the AFP in females are connected in a similar manner as has been shown for males, for which the AFP plays an important role in the development of song and maintenance in adulthood. We assume that females that do not learn to sing also make use of this pathway, but adopt it for their special needs of memorization and recognition tasks. Identifying the AFP in female birds furthermore gives evidence for the preservation of the cortico-basal ganglia thalamocortical pathway in mammals and birds.

**REFERENCES**


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