available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Characterization of CaMKII-expressing neurons within a striatal region implicated in avian vocal learning

A.M. Hein[1], *A. Sridharan*, *K.W. Nordeen**, *E.J. Nordeen*

Department of Brain & Cognitive Sciences, University of Rochester, Rochester, NY 14627, USA

ARTICLE INFO

Article history:
Accepted 7 April 2007
Available online 19 April 2007

Keywords:
CaMKII
DARPP-32
Neurogenesis
Striatum
Medium spiny neurons
Songbird

ABSTRACT

In songbirds, an anterior forebrain pathway has been implicated in vocal learning. Within Area X, the striatal–pallidal component of this forebrain pathway, early social tutoring dramatically increases the autophosphorylation of CaMKII (calcium-calmodulin-dependent protein kinase II). Activation of CaMKII often is associated with forms of synaptic plasticity (e.g. LTP) underlying learning and memory, and electrophysiological studies have demonstrated NMDA and dopamine (DA) receptor-dependent LTP among Area X medium spiny neurons [Ding, L., Perkel, D.J., 2002. Dopamine modulates excitability of spiny neurons in the avian Basal Ganglia. J. Neurosci. 22, 5210–5218]. Together, these data suggest that Area X neurons may help to encode a representation of song used for vocal mimicry. To identify which Area X neurons could participate in the CaMKII response to song tutoring, we used immunocytochemistry to assess the colocalization of CaMKII with several other biochemical markers that identify specific neuron classes within Area X. Virtually all (~98%) Area X cells expressing CaMKII also expressed DARPP-32 (dopamine- and adenosine 3′5′-monophosphate-regulated phosphoprotein), a dopamine signaling protein enriched in medium spiny striatal neurons. The implication that medium spiny neurons are primary mediators of the pCaMKII response to tutoring is interesting in view of the established dopaminergic modulation of LTP in this cell type. Additionally, BrdU and DARPP-32 immunocytochemistry were combined to test whether medium spiny neurons are among the neurons generated and incorporated into Area X during song learning. Based upon their expression of DARPP-32, the majority of Area X neurons labeled by BrdU injections given on posthatch days 20–25 are medium spiny neurons.

1. Introduction

In songbirds, Area X is a specialized portion of the avian basal ganglia that comprises part of an anterior forebrain pathway (AFP) critical for normal song learning in juveniles, as well as vocal plasticity in adulthood (Bottjer et al., 1984; Brainard and Doupe, 2000; Scharff and Nottebohm, 1991; Sohrabji et al., 1990; Williams and Mehta, 1999). Lesioning Area X in juvenile male zebra finches prevents the development of a stable song pattern (Scharff and White, 2004; Sohrabji et al., 1990), suggesting that this region may play a role in encoding song-related auditory memories used for vocal imitation (sensory acquisition), and/or in comparing vocal output to auditory memories during vocal practice (sensorimotor learning). Interestingly, although Area X lesions made in adult male zebra finches have little impact on the production of

* *Corresponding author*. Fax: +1 585 442 9216.
E-mail address: knordeen@bcs.rochester.edu (K.W. Nordeen).
[1] Current address: Department of Psychology and Center for Neuroscience, University of Colorado at Boulder, Boulder, CO 80309, USA.

0006-8993/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2007.04.025

stereotyped song, singing is associated with motor-related activity (Hessler and Doupe, 1999) and an increase in immediate early gene expression (Jarvis and Nottebohm, 1997; Jarvis et al., 1998) within Area X, suggesting that this region may also participate in song maintenance. With respect to song learning, several key observations support a role for Area X in sensory acquisition. First is the observation that neurons in this region respond both to tutor song and the bird's own song (Solis and Doupe, 1999). Furthermore, song tutoring of juvenile songbirds promotes phosphorylation of calcium-calmodulin-dependent protein kinase II (CaMKII), an event that in other systems has been linked to synaptic strengthening and behavioral learning (Singh et al., 2005). This effect of song tutoring occurs in the absence of vocal practice and only under stimulus conditions that are optimal for vocal imitation. Moreover in zebra finches, it occurs in males (who learn to sing) but not in females (who do not sing), suggesting that it is not a generalized striatal response to hearing song, but rather that the tutoring-induced CaMKII activation requires and/or affects male-specific circuitry related to vocal learning.

Because of the pivotal role that CaMKII phosphorylation plays in many instances of synaptic strengthening, locating the specific neurons expressing this molecular marker could point to where such synaptic strengthening might occur within Area X during song learning. Several observations suggest that medium spiny neurons within Area X are likely to participate in the CaMKII response to tutoring. First, these small GABAergic neurons are estimated to constitute about 80% of the neurons within Area X (Reiner et al., 2004). Second, long-term potentiation (LTP) within Area X occurs at the glutamatergic synapses formed on medium spiny neurons (Ding and Perkel, 2004). These glutamatergic inputs arise from both HVC (acronym used as proper name), a region implicated in song production that conveys auditory information into the AFP, and from LMAN (lateral magnocellular nucleus of the anterior nidopallium), another component of the AFP implicated specifically in vocal learning and plasticity. Third, dopamine receptor activation is required to induce LTP of the glutamatergic inputs to medium spiny neurons in Area X (Ding and Perkel, 2004), and also facilitates the tutoring-induced phosphorylation of CaMKII within this region (unpublished data). Medium spiny neurons receive strong dopaminergic innervation from the ventral tegmental area and substantia nigra, and they express DARPP-32, a dopamine signaling molecule that has been implicated in the modulation of striatal synaptic plasticity (Bottjer, 1993; Ding and Perkel, 2002; Ding et al., 2003; Lewis et al., 1981; Reiner et al., 2004; Soha et al., 1996).

In the present study, medium spiny neurons in Area X were identified by their expression of DARPP-32 (Ouimet et al., 1984; Reiner et al., 2004; Reiner et al., 1998), and double labeling experiments using an antibody directed against CaMKII

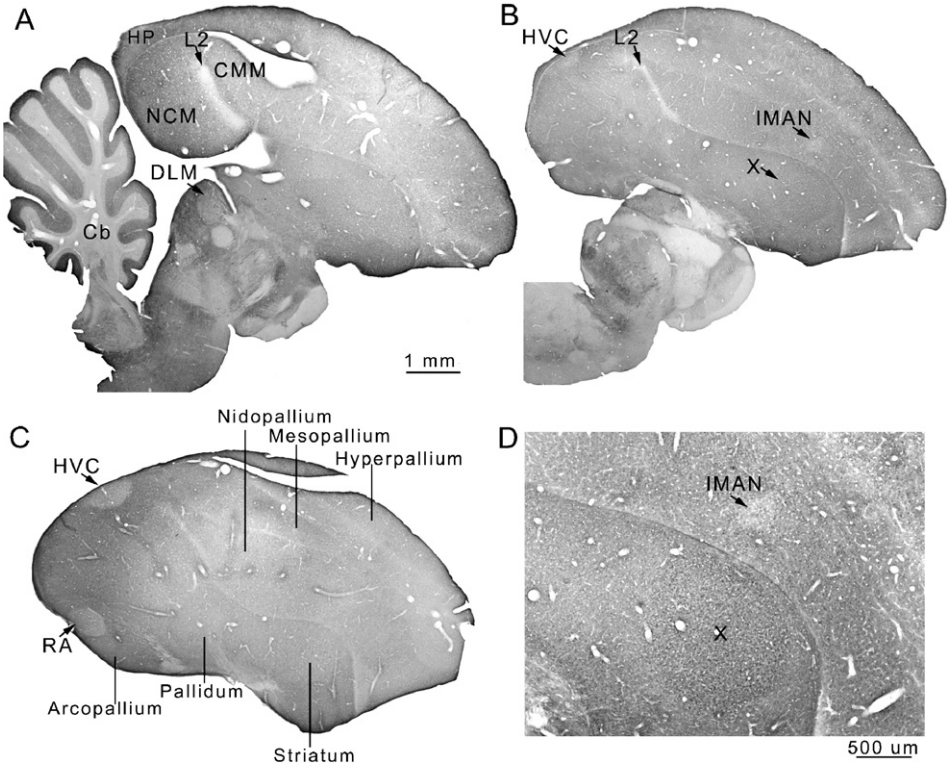

Fig. 1 – CaMKII immunoreactivity within sagittal sections of a juvenile male zebra finch. Low power images progress from medial (A) to lateral (C). Panel D (higher power image taken from the same section as shown in B) shows that CaMKII immunoreactivity within Area X is higher than in surrounding striatum. Abbreviations: Cb, cerebellum; CMM, caudomedial mesopallium; DLM, medial nucleus of the dorsolateral thalamus; HP, hippocampus; HVC (acronym is proper name); L2, Field L2; LMAN, lateral magnocellular nucleus of anterior nidopallium; NCM, caudomedial nidopallium; RA, robust nucleus of arcopallium; X, Area X.

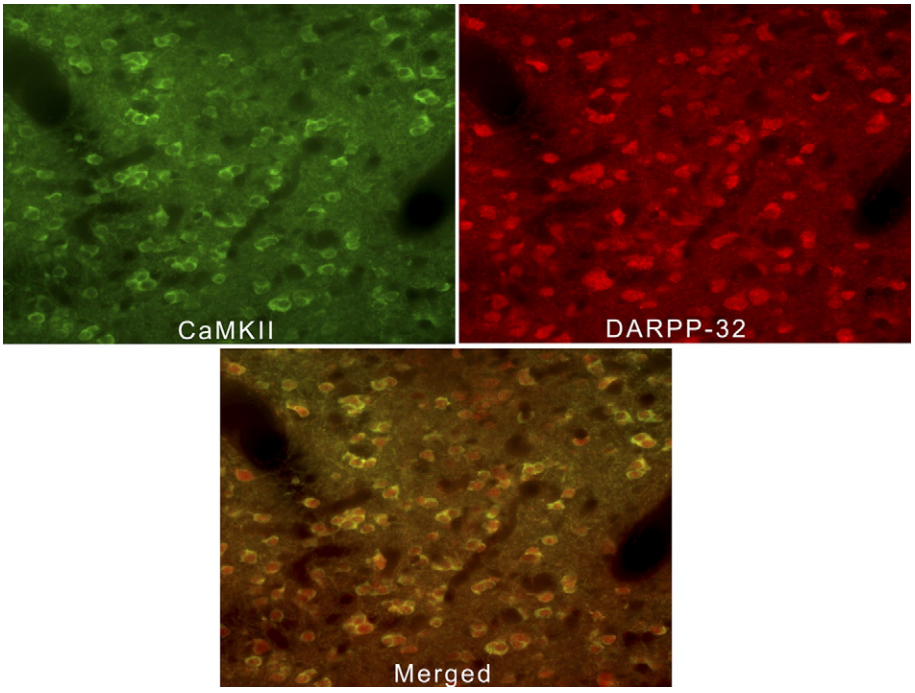

Fig. 2 – Photomicrographs of a single field of Area X neurons showing colocalization of CaMKII and DARPP-32. Virtually all of the neurons that expressed CaMKII also expressed DARPP-32.

revealed nearly complete overlap in the expression of DARPP-32 and CaMKII immunoreactivity. CaMKII immunoreactivity was sparse or absent from two other Area X neuronal subpopulations that can be identified by neurochemical criteria (Reiner et al., 2004): (1) large neurons expressing the neurotensin-related hexapeptide LANT6 (Lys8-Asn9-neurotensin8–13), and (2) a subclass of striatal interneurons expressing parvalbumin (PARV).

We also evaluated whether these medium spiny neurons comprise a significant proportion of the neurons recruited to Area X during song learning (Kirn and DeVoogd, 1989; Nordeen and Nordeen, 1988). We previously established only that the neuronal cohorts recruited to Area X during adolescence do not include the major pallidal neuron type that projects to the thalamus (Sohrabji et al., 1993). In the present report we find that many of these newly recruited striatal neurons in young males are medium spiny neurons.[2] Together the results suggest that song learning may provoke synaptic plasticity even among recently recruited striatal neurons, perhaps directly affecting the developmental organization of Area X.

2. Results

2.1. *Identification of CaMKII-expressing Area X neurons*

Total CaMKII immunoreactivity was evident throughout much of the zebra finch brain (Fig. 1). Within the telencephalon, immunolabeling was evident within both perikarya and neuropil of hippocampus, caudal nidopallium, arcopallium, striatum, and pallidum. Only slightly lower levels of CaMKII immunoreactivity were present in mesopallium and the remainder of nidopallium. Immunoreactivity in the dorsal thalamus was relatively high; in other portions of thalamus and brainstem it varied markedly across nuclei. Within the cerebellum, granule cells were lightly labeled while moderate levels of CaMKII immunoreactivity were evident in Purkinje cells.

Among the telencephalic song and auditory nuclei, CaMKII immunoreactivity varied dramatically. Qualitative inspection revealed CaMKII expression levels within Area X that were relatively high compared to other song regions. Neuropil labeling appeared similar within and outside of Area X, but the intensity of somal labeling appeared somewhat greater within Area X than in the surrounding medial striatum (Fig. 1D). There was no obvious evidence of regional heterogeneity in CaMKII expression within Area X. Within the nidopallium, the LMAN and HVC were both clearly identifiable due to much lower CaMKII immunoreactivity as compared to the surrounding tissue (Figs. 1B, C, and D). Also, within the robust nucleus of the arcopallium (RA), another nucleus necessary for song production, overall expression of CaMKII was notably less than in surrounding arcopallium (Fig. 1C). Among telencephalic auditory regions, Field L2 was clearly discernable due to reduced CaMKII immunoreactivity relative to surrounding tissue (Figs. 1A and B), while CaMKII expression was moderate in CMM (caudomedial mesopallium) and moderate–high in NCM (caudomedial nidopallium).

Within Area X, the vast majority of CaMKII+ cells were identified as medium spiny neurons based upon their coexpression of DARPP-32. CaMKII+ perikarya were abundant in Area X, were relatively small in diameter, and virtually all

[2] Since the submission of this article, Scott and Lois (2007) employed retroviral GFP labeling and morphological criteria to conclude similarly that most Area X neurons generated during adolescence are medium spiny neurons.

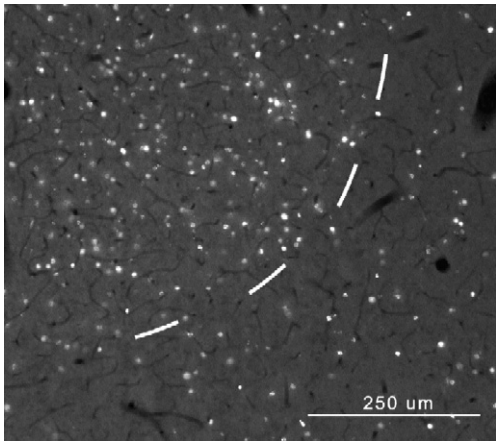

Fig. 3 – Photomicrograph of (coronal section) showing the density of BrdU+ cells within Area X is greater than in the surrounding striatum. The dashed line shows the medial ventral boundary of Area X; medial is to the right.

(98%±1%) were DARPP-32+. Similarly, DARPP-32+ cells were abundant in Area X, were of relatively small diameter, and the large majority (93%±2%) colabeled for CaMKII. Representative photomicrographs in Fig. 2 illustrate this extensive colocalization of CaMKII and DARPP-32.

In contrast to the immunocytochemical overlap seen between CaMKII and DARPP-32 expression, we found relatively little CaMKII immunoreactivity in either the large LANT6+ neurons, or in PARV+ neurons. We detected intense LANT6 immunoreactivity within Area X perikarya that were notably larger and less abundant than the population of DARPP-32+ cells. An average of 43 of these large LANT6+ cells were evaluated within Area X in each bird, and none of these cells expressed any detectable immunoreactivity for CaMKII. Also, PARV+ perikarya in Area X were notably less abundant than DARPP-32+ cells, and relatively few PARV+ perikarya expressed immunoreactivity to the pCaMKII antibody. An average of 139 PARV+ neurons were evaluated in each animal, and in only two animals were any of these cells found to be pCaMKII+. Overall, 3%±2% of the PARV+ cells coexpressed pCaMKII. We would caution against accepting the paucity of CaMKII labeling among these LANT6+ and PARV+ cells as proof that they do not express CaMKII. However, it is important to reiterate that virtually all (98%) of the CaMKII+ cells we observed were also DARPP-32+, and other studies have concluded on the basis of their size, distribution, and/or a spiny morphology that both the PARV+ and large LANT6+ cell populations are distinct from the DARPP-32+ striatal cells (Farries and Perkel, 2002; Kawaguchi, 1993; Reiner et al., 2004).

2.2. *Characterization of Area X neurons generated during adolescence*

BrdU labeling revealed significant regional variation in the incorporation and/or survival of striatal cells generated post-hatch. While BrdU+ cells were present throughout the striatum, labeling was visibly greater within Area X (Fig. 3). The density of BrdU+ cells within Area X was approximately 2.5× greater than that in the striatum immediately ventral to Area X ($t=12.60$, $df=3$, $p<0.001$; see Fig. 4). Within Area X, we did not detect any obvious heterogeneity in the density of BrdU+ cells.

Colabeling experiments revealed that many of the BrdU+ cells within Area X were also immunoreactive for DARPP-32 (Fig. 5). Moreover, the percentage of BrdU+ cells that were also DARPP-32+ was significantly greater within Area X than in the region ventral to Area X ($t=7.838$, $df=3$, $p<0.005$; see Fig. 6). In Area X, approximately 60% of the BrdU+ cells coexpressed DARPP-32, whereas in the surrounding striatum only 40% of the BrdU+ cells were immunoreactive to the DARPP-32 antibody.

Although both BrdU+ cells and BrdU+/DARPP-32+ cells were enriched within Area X, no regional differences were detected in the percentage of BrdU+ cells classified as neurons based on their expression of the Hu protein (Fig. 6). Within Area X, 75% (±5.5%) of the BrdU+ cells were Hu+. Similarly, 77% (±1.8%) of the BrdU cells in the striatal region outside Area X were colabeled with Hu.

3. Discussion

Social tutoring promotes a robust increase in the phosphorylation of CaMKII within Area X of juvenile male zebra finches, a biochemical response not seen in similarly tutored females (Singh et al., 2005). Here we show that virtually all of the Area X neurons that express CaMKII immunoreactivity in juvenile male zebra finches are medium spiny neurons, identified by their coexpression of DARPP-32 immunoreactivity. These data are consistent with studies in rat that concluded from colocalization of calcineurin and CaMKII that most CaMKII+ neurons within the striatum are medium spiny neuron (Goto et al., 1994). Also, we provide evidence that many of the neurons born and recruited to Area X in males during vocal learning express DARPP-32. Together, the present results

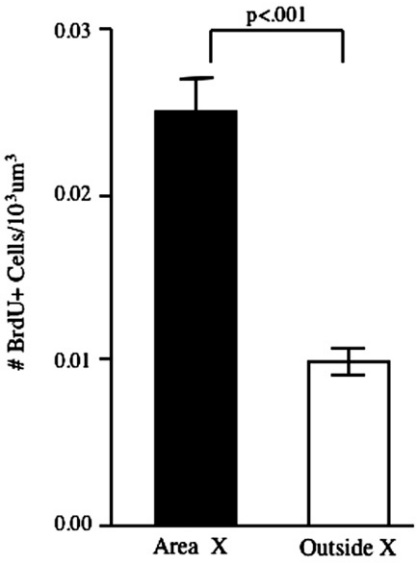

Fig. 4 – Histograms showing the density of BrdU+ cells within Area X and in striatum outside (ventral to) Area X. Significantly more BrdU+ cells were present inside Area X than in the surrounding striatum. Data shown are mean±SEM.

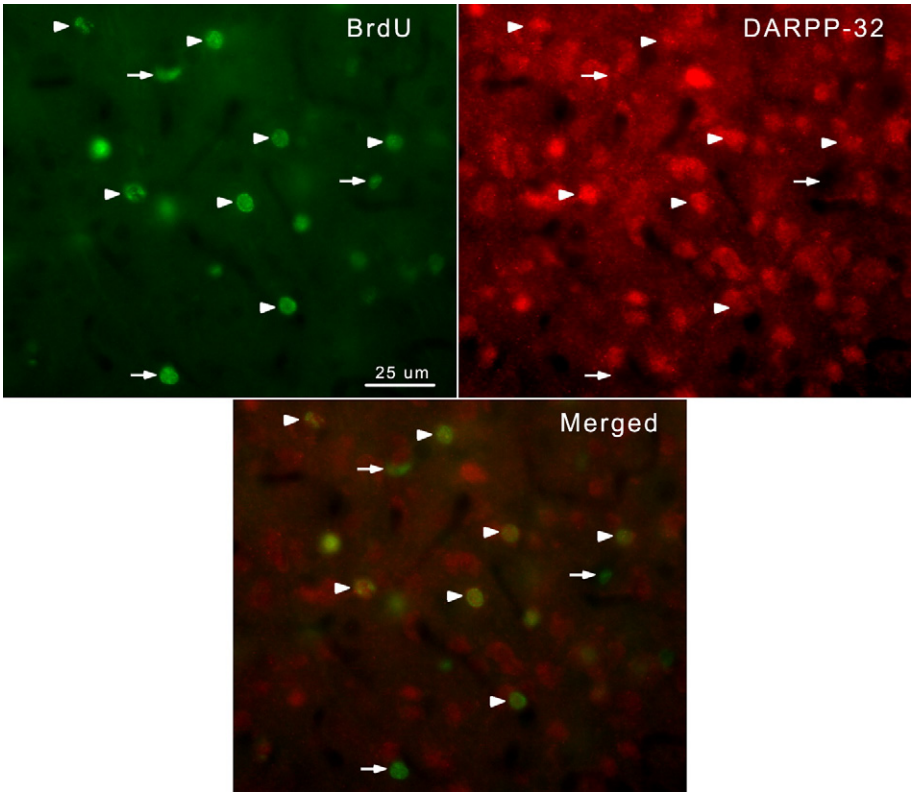

Fig. 5 – Photomicrographs of a single field of Area X neurons showing colocalization of BrdU and DARPP-32. Large arrowheads identify cells that were BrdU+ and DARPP-32+. Smaller arrows identify cells that were BrdU+ but did not express detectable DARPP-32 immunoreactivity.

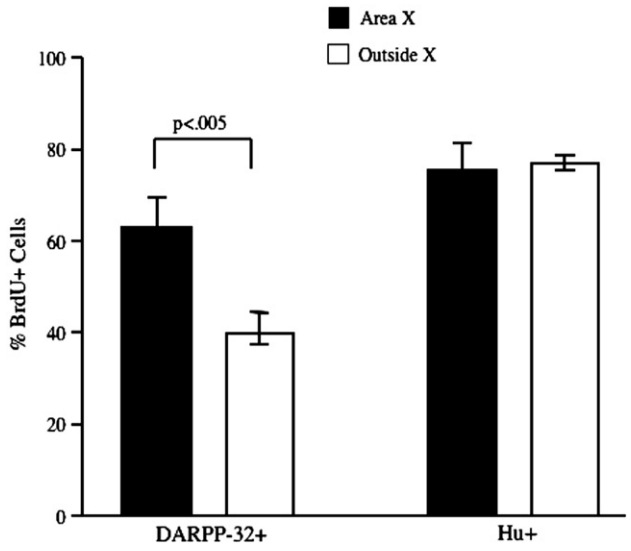

Fig. 6 – Histograms showing the percentage of BrdU+ cells inside and outside (ventral to) Area X that also stained positive for DARPP-32 or Hu. While the proportion of BrdU+ cells that were also immunoreactive for the Hu protein was similar inside and outside Area X, significantly more BrdU+ cells were DARPP-32+ within Area X as compared to outside Area X. Data shown are mean ± SEM.

suggest that DARPP-32 expressing medium spiny neurons recruited to Area X during the juvenile period may, in addition to whatever role they play in sensorimotor learning, participate in establishing a representation of tutor song that is used to guide vocal motor development.

We have proposed previously that tutoring-induced CaMKII phosphorylation within Area X may promote specific forms of synaptic plasticity (e.g. LTP) important for song learning. This hypothesis has not been tested directly, but is bolstered by the present observation that CaMKII in Area X is localized primarily to medium spiny neurons. Glutamatergic inputs (from HVC and LMAN) onto this striatal cell class do exhibit LTP in male zebra finches, and this synaptic plasticity requires dopamine receptor activation (Ding and Perkel, 2004), as does the tutoring-induced elevation in pCaMKII we have described within Area X (Hein et al., 2005). The expression of DARPP-32 by medium spiny neurons could provide the basis for this dopaminergic regulation of LTP in Area X. D1 receptor signaling promotes phosphorylation of DARPP-32 on Thr34, which results in powerful inhibition of protein phosphatase 1 (Svenningsson et al., 2004). Since this phosphatase inactivates CaMKII and negatively regulates several other proteins involved in the establishment and maintenance of LTP (Mansuy and Shenolikar, 2006), DARPP-32 inhibition of protein phosphatase 1 favors the various processes and signaling cascades involved in synaptic strengthening (Genoux et al., 2002; Lisman and Zhabotinsky, 2001; Soderling and Derkach, 2000). To further assess the hypothesis that medium spiny

neurons are a site of synaptic change related to stimulus encoding during song acquisition, it will be important to demonstrate that tutoring-induced increases in pCaMKII within Area X are accompanied by phosphorylation of Thr34–DARPP-32 within these striatal neurons.

Another important aspect of this study is the confirmation and extension of earlier work characterizing Area X neuronal recruitment during vocal learning. In this earlier work (Sohrabji et al., 1993), our assessment of neuronal recruitment into Area X relied on morphological criteria applied to Nissl-stained tissue, so it is important that, in the present report, Hu immunohistochemistry confirmed that most cells recruited into Area X during early adolescence are indeed neuronal and that the insertion and/or survival of neurons born between 20 and 25 days posthatch is greater within Area X than in the surrounding medial striatum. Importantly, the results presented here show also that medium spiny neurons are the majority cell type born and added to Area X during this developmental period. In a recent report, Scott and Lois concluded similarly using retroviral GFP labeling and morphological criteria for cell identification (Scott and Lois, 2007). In this report, medium spiny neuronal phenotype was further confirmed by DARPP-32 immunoreactivity in six out of seven GFP+ cells.

Although DARPP-32 expression is a reliable marker for medium spiny striatal neurons, this neuronal class is itself heterogeneous and includes both substance P-expressing and enkephalinergic neurons (Graybiel, 1990; Reiner et al., 1998). Thus, further work is needed to determine whether striatal neurogenesis during adolescence includes all types of medium spiny neuron or just a subclass of such cells. Also, while an earlier report indicated that Area X neurons projecting to thalamus are not among those added to Area X in young birds (Sohrabji et al., 1993), we cannot rule out the possibility that a small number of other striatal cell types also are recruited during adolescence. However, within the timeframe examined, such recruitment would have to be sparse since the newly generated neurons within Area X (as indicated by Hu labeling) are almost totally accounted for by the percentage of BrdU cells expressing DARPP-32.

Some interesting regional differences in neuronal recruitment were observed in this study. For instance, the recruitment of newly generated neurons was greater within Area X than in the surrounding striatum. Furthermore, among those neurons labeled by our BrdU treatment, DARPP-32+ perikarya were enriched within Area X as compared to the immediately surrounding striatum. Yet, the overall density of DARPP-32+ cells in our tissue was not obviously different between Area X and the surrounding medial striatum. Likewise, a previous study of adult male zebra finches noted only slightly greater DARPP-32 immunoreactivity in the neuropil and perikarya within Area X than in surrounding striatum (Reiner et al., 2004), and in starlings D1 dopamine receptor binding is only slightly higher within Area X as compared to adjacent striatal regions (Casto and Ball, 1994). While recent evidence indicates that both medium spiny neurons destined for Area X and those recruited into the surrounding striatum arise from the same region of the ventricular wall (a region sharing molecular similarity to the medial and lateral ganglionic eminences in mammals), the progenitor pools for these two populations of neurons may have different developmental patterns of cell division (Scott and Lois, 2007). Alternatively, the relatively greater incidence of BrdU+ and DARPP-32+/BrdU+ cells within Area X could arise through regional differences in the survival or specification of the medium spiny neuron population within this specialized striatal region. Indeed, Kirn and DeVoogd have shown marked developmental and regional differences in the rates of neuronal recruitment and survival within Area X and adjacent regions of the striatum (Kirn and DeVoogd, 1989). Differences also could arise through afferents that specifically target Area X. In males, Area X receives glutamatergic input from pallial regions HVC and LMAN (regions implicated in song production and song learning, respectively). These afferents could secondarily influence the survival of their target neurons within Area X, as has been shown to occur in the RA, another song nucleus that receives afferents from HVC and LMAN (Burek et al., 1995; Johnson and Bottjer, 1994). Also, regional differences could stem from singing-related activity being higher within Area X than in the surrounding striatum since singing is associated with increased neuron survival within HVC, another song-related region (Li et al., 2000). Another possibility is that regional differences in neuronal recruitment could stem from differences in dopaminergic innervation since projections from ventral tegmental area and substantia nigra to the avian striatum appear to be especially dense within Area X (Bottjer, 1993; Lewis et al., 1981; Soha et al., 1996). However, this seems less likely since this disproportionate pattern of catecholaminergic innervation does not emerge until relatively late in the juvenile period (Soha et al., 1996).

Studies of the mammalian striatum indicate that neurogenesis among medium spiny neurons is largely complete by birth. For instance, in rodents, such cells are postmitotic by postnatal day 2, with their peak of neurogenesis occurring around embryonic day 15 (Bayer, 1984; Fentress et al., 1981; Marchand and Lajoie, 1986). Yet neural stem cells within the lateral ganglionic eminence clearly retain the ability to generate striatal neurons into adulthood. Modest striatal neurogenesis has been reported in adult rodent (Dayer et al., 2005) and non-human primate (Bedard et al., 2006), although the neuron subpopulation(s) involved were not identified in these studies. Significantly, striatal damage sustained in adulthood can stimulate the recruitment of new striatal neurons, many of which express the chemical phenotype of medium spiny neurons (Arvidsson et al., 2002; Collin et al., 2005; Jin et al., 2003; Parent et al., 2002). In addition, overexpression of brain-derived neurotrophic factor can stimulate the genesis of medium spiny neurons in adult rodents (Benraiss et al., 2001; Chmielnicki et al., 2004) and non-human primates (Bedard et al., 2006), and this effect can be potentiated by concurrent overexpression of noggin, an inhibitor of glial differentiation (Benraiss et al., 2001; Chmielnicki et al., 2004). The developmental expression patterns of these molecules have not been studied in relation to the posthatching recruitment of Area X cells, but BDNF may be especially interesting since it has been implicated in promoting neuronal recruitment to the song region HVC in juvenile and adult birds (Rasika et al., 1999).

The identification of a neuronal type in Area X that likely participates in tutoring-induced synaptic plasticity will help us understand how circuitry in this specialized striatal region

contributes to the capacity for song behavior. In addition, the ongoing recruitment of large numbers of these cells during the normal period of song acquisition could encourage the creation of neural circuits that encode template information used later for vocal mimicry. These and other studies characterizing the developmental history of this striatal cell type may help in developing approaches to selectively manipulate circuits controlling song to elucidate their role in vocal learning.

4. Experimental procedure

Animal protocols were approved by the University of Rochester Committee on Animal Resources and conform to NIH guidelines. All experiments involved male zebra finches (*Taeniopygia guttata*) that were hatched and raised in our laboratory and maintained on a 14:10 hour light:dark cycle.

4.1. *Identification of CaMKII-expressing Area X neurons*

To characterize the cell types in Area X that express CaMKII, birds were reared and tutored as in previous studies documenting CaMKII activation in this region following song tutoring (Singh et al., 2005). Briefly, juveniles were removed from their parents and clutchmates at posthatch day 30 and individually isolated in a soundproof chamber. At the beginning of the light cycle on posthatch day 35, each juvenile male was placed into its father's cage, which was located behind a unidirectional microphone interfaced to a sound-activated recording system (Avisoft-Recorder). An adult female was placed in an adjacent cage to encourage song production by the tutor. A human observer monitored a real-time spectrographic display to note when the tutor first sang (typically, this was immediately after the lights were illuminated), and juveniles were sacrificed 2 h after the onset of tutor song. Pupils did not produce any subsong during the tutoring session.

At the conclusion of the tutoring session, juveniles ($n=6$) were anesthetized with an overdose of barbiturate anesthesia and transcardially perfused with cold 1 M sodium phosphate buffer (PB; pH 7.4) containing .9% saline and .5% sodium nitrate, followed by 4% paraformaldehyde (PFA) and 2.5% gluteraldehyde in PB. Brains were removed, postfixed for 2–4 h, placed in 30% sucrose in .9% phosphate buffered saline (PBS) overnight at 4 °C, and then frozen and sectioned (50 μm, sagittal) on a sliding microtome. Every third section was stained with thionin, and the remaining sections were stored at −20 °C in cryoprotectant (30% ethylene glycol and 30% sucrose in PB).

In order to assess what specific cell types within Area X could underlie the tutor induced CaMKII activation described in earlier work (Singh et al., 2005), we assessed the colocalization of CaMKII with antigens that define specific cell types within Area X (Reiner et al., 2004). Sections were washed in PBS containing .3% Triton (PBS/TX) for 6×10 min, preblocked in 10% normal goat serum (NGS: Vector Laboratories) for 30 min, and then incubated in primary antibody at 4 °C. For colocalization studies focused on the DARPP-32 and LANT6 neuronal populations, we combined anti-DARPP-32 (Cell Signaling Technologies, 1:500 rabbit polyclonal) or anti-LANT6 (courtesy of Dr. Carraway; 1:500 rabbit polyclonal) with an anti-CaMKII antibody (Sigma; 1:1000 mouse monoclonal) that recognizes both unphosphorylated and phosphorylated CaMKII. In assessing colocalization among the PARV+ interneuron population we combined anti-PARV (Sigma; 1:10,000 mouse monoclonal) with an anti-pCaMKII antibody (Promega; 1:300 rabbit polyclonal) in order to avoid problems arising from the use of primary antibodies raised within the same species. This pCaMKII antibody recognizes CaMKII phosphorylated on threonine 286 and does not bind to the non-phosphorylated form of CaMKII (see Singh et al., 2005). Pilot studies in our laboratory established that all Area X cells that are immunoreactive to the CaMKII antibody also expressed detectable immunoreactivity to the pCaMKII antibody.

After overnight incubation in primary antibodies (4 night incubation for the pCaMKII and PARV antibodies), washes and preblocking steps were repeated followed by a 4-hour incubation at room temperature in goat anti-rabbit Alexa 555 and anti-mouse Alexa 488 (Molecular Probes; 1:800). Sections then were washed thoroughly in PBS/TX followed by PBS (2 h), mounted, coverslipped with VectaShield (Vector Laboratories), and stored in the dark at 4 °C.

Immunoreactivity was visualized using an Olympus AX70 fluorescence microscope, Olympus MicroFire 1.0 camera, and Image Pro software (5.0). Immunoreactive cells that had distinct cellular boundaries were manually counted for each of the antibodies. For colocalization analysis among DARPP-32+ medium spiny neurons, the total number of immunoreactive cells within a 148 μm×196 μm field within Area X was counted at 40×. Two such fields (dorsal/anterior and ventral/posterior) were counted in each animal. No regional differences in immunoreactivity within Area X were detected so counts from these 2 areas were averaged. The total number of cells immunoreactive for CaMKII or DARPP-32 was counted separately and then the number of colabeled cells was determined. Because large LANT6+ and PARV+ cells are relatively sparse in Area X (see also Reiner et al., 2004), the entirety of Area X was scanned for cells expressing these proteins and for each such cell encountered colabeling for CaMKII (or pCaMKII in the case of PARV) was assessed.

4.2. *Characterization of Area X neurons generated during adolescence*

Juvenile male zebra finches were housed with their parents and clutchmates throughout the duration of the experiment. From posthatch day 20 to 24, they were injected intramuscularly twice daily with BrdU (Sigma, 50 mg/kg, 15 mg/ml). Thirty days after the last BrdU injection (sufficient time for new neurons to migrate to and differentiate within Area X), birds were anesthetized and perfused as described above. Brains were postfixed in PFA, sunk in 30% sucrose solution overnight at 4 °C, and frozen and serially sectioned (30 μm, coronal) on a sliding microtome. Sections were stored at −20 °C in cryoprotectant.

For immunocytochemical detection of BrdU and DARPP-32, free floating sections were first washed in 0.05 M PBS 9×10 min. To denature DNA and expose the BrdU epitope for the antibody, the following pretreatment steps were carried out: incubation in 50% formamide/2× SSC buffer (standard

sodium citrate; 1× SSC=0.15 M NaCl and 0.015 M sodium citrate dihydrate, pH 7.0) at 65 °C for 2 h, rinse in 2× SSC buffer twice for 5 min, incubation in 2 N HCl for 30 min at 37 °C, and rinse in 0.1 M borate buffer (0.1 M boric acid and 0.025 M borax; pH 8.5) for 10 min. Following a 1-h blocking step in 5% NGS/0.3% TX/1% bovine serum albumin (BSA) in PBS, sections were incubated in anti-BrdU (Becton Dickinson Immunocytometry Systems; 1:500 mouse monoclonal) and anti-DARPP-32 overnight at 4 °C. The washes and blocking step were then repeated followed by incubation in a pooled solution of Alexa 555-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-mouse for 4 h. Sections then were rinsed in PBS for 2 h, mounted, coverslipped using VectaShield, and stored in the dark at 4 °C.

Double labeling immunofluorescence studies of BrdU and Hu were performed in a similar way, except that the primary incubations were done sequentially. Briefly, sections were first run through DNA denaturation steps, then blocked and incubated in anti-Hu (Molecular Probes; reconstituted at 100 μg/ml in PBS containing 1% BSA; 1:50 mouse monoclonal) overnight at 4 °C. After additional washes and blocking, sections were incubated for 2 h in biotinylated goat anti-mouse secondary antibody (Vector Laboratories, 1:100). Sections were then thoroughly washed, incubated in Avidin Texas Red (Vector Laboratories; 1:50), washed and blocked again, and incubated in anti-BrdU (Serotec; 1:10 rat) overnight at 4 °C. The washes and blocking steps were then repeated and followed by incubation for 2 h at RT with Alexa 488-conjugated goat anti-rat (Molecular Probes; 1:400).

Immunoreactivity was visualized as described above. In each of two sections (one anterior, one posterior) from each animal ($n=4$), two 148 μm × 196 μm fields within the boundaries of Area X and three fields located between the ventral border of Area X and the ventral surface of the brain were analyzed. Although the boundaries of Area X were evident based on the density of BrdU+ cells (see Results), adjacent sections that were stained with thionin were used to confirm the identification of these boundaries. For both BrdU/DARPP-32 and BrdU/Hu analyses, the number of BrdU labeled cells was first counted within the sample field and then the proportion of these cells that were DARPP-32 or Hu labeled was determined. For BrdU/DARPP-32 and BrdU/Hu labeling, paired *t*-tests (two-tailed) evaluated differences between inside and outside Area X.

Acknowledgments

We are grateful for expert technical support from Heather Bradstreet and Dr. David Holtzman. We also thank Dr. Robert Carraway for his generous gift of the LANT6 antibody. Supported by NIH MH068546.

REFERENCES

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., Lindvall, O., 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat. Med. 8, 963–970.

Bayer, S.A., 1984. Neurogenesis in the rat neostriatum. Int. J. Dev. Neurosci. 2, 163–175.

Bedard, A., Gravel, C., Parent, A., 2006. Chemical characterization of newly generated neurons in the striatum of adult primates. Exp. Brain Res. 170, 501–512.

Benraiss, A., Chmielnicki, E., Lerner, K., Roh, D., Goldman, S.A., 2001. Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. J. Neurosci. 21, 6718–6731.

Bottjer, S.W., 1993. The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches. J. Neurobiol. 24, 51–69.

Bottjer, S.W., Miesner, E.A., Arnold, A.P., 1984. Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science 224, 901–903.

Brainard, M.S., Doupe, A.J., 2000. Interruption of a basal ganglia–forebrain circuit prevents plasticity of learned vocalizations. Nature 404, 762–766.

Burek, M.J., Nordeen, K.W., Nordeen, E.J., 1995. Initial sex differences in neuron growth and survival within an avian song nucleus develop in the absence of afferent input. J. Neurobiol. 27, 85–96.

Casto, J.M., Ball, G.F., 1994. Characterization and localization of D1 dopamine receptors in the sexually dimorphic vocal control nucleus, area X, and the basal ganglia of European starlings. J. Neurobiol. 25, 767–780.

Chmielnicki, E., Benraiss, A., Economides, A.N., Goldman, S.A., 2004. Adenovirally expressed noggin and brain-derived neurotrophic factor cooperate to induce new medium spiny neurons from resident progenitor cells in the adult striatal ventricular zone. J. Neurosci. 24, 2133–2142.

Collin, T., Arvidsson, A., Kokaia, Z., Lindvall, O., 2005. Quantitative analysis of the generation of different striatal neuronal subtypes in the adult brain following excitotoxic injury. Exp. Neurol. 195, 71–80.

Dayer, A.G., Cleaver, K.M., Abouantoun, T., Cameron, H.A., 2005. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J. Cell Biol. 168, 415–427.

Ding, L., Perkel, D.J., 2002. Dopamine modulates excitability of spiny neurons in the avian basal ganglia. J. Neurosci. 22, 5210–5218.

Ding, L., Perkel, D.J., 2004. Long-term potentiation in an avian basal ganglia nucleus essential for vocal learning. J. Neurosci. 24, 488–494.

Ding, L., Perkel, D.J., Farries, M.A., 2003. Presynaptic depression of glutamatergic synaptic transmission by D1-like dopamine receptor activation in the avian basal ganglia. J. Neurosci. 23, 6086–6095.

Farries, M.A., Perkel, D.J., 2002. A telencephalic nucleus essential for song learning contains neurons with physiological characteristics of both striatum and globus pallidus. J. Neurosci. 22, 3776–3787.

Fentress, J.C., Stanfield, B.B., Cowan, W.M., 1981. Observation on the development of the striatum in mice and rats. Anat. Embryol. (Berl) 163, 275–298.

Genoux, D., Haditsch, U., Knobloch, M., Michalon, A., Storm, D., Mansuy, I.M., 2002. Protein phosphatase 1 is a molecular constraint on learning and memory. Nature 418, 970–975.

Goto, S., Yamada, K., Oyama, T., Korematsu, K., Nagahiro, S., Ushio, Y., Fukunaga, K., Miyamoto, E., Hofer, W., 1994. Cellular localization of type II Ca2+/calmodulin-dependent protein kinase in the rat basal ganglia and intrastriatal grafts derived from fetal striatal primordia, in comparison with that of Ca2+/calmodulin-regulated protein phosphatase, calcineurin Neuroscience 62, 695–705.

Graybiel, A.M., 1990. Neurotransmitters and neuromodulators in the basal ganglia. Trends Neurosci. 13, 244–254.

Hein, A.M., Hart, A.S., Nordeen, K.W., Nordeen, E.J., 2005. Dopamine signaling may contribute to tutoring-induced CaMKII activation within the avian basal ganglia.

Hessler, N.A., Doupe, A.J., 1999. Singing-related neural activity in a dorsal forebrain–basal ganglia circuit of adult zebra finches. J. Neurosci. 19, 10461–10481.
Jarvis, E.D., Nottebohm, F., 1997. Motor-driven gene expression. Proc. Natl. Acad. Sci. U. S. A. 94, 4097–4102.
Jarvis, E.D., Scharff, C., Grossman, M.R., Ramos, J.A., Nottebohm, F., 1998. For whom the bird sings: context-dependent gene expression. Neuron 21, 775–788.
Jin, K., Sun, Y., Xie, L., Peel, A., Mao, X.O., Batteur, S., Greenberg, D.A., 2003. Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Mol. Cell. Neurosci. 24, 171–189.
Johnson, F., Bottjer, S.W., 1994. Afferent influences on cell death and birth during development of a cortical nucleus necessary for learned vocal behavior in zebra finches. Development 120, 13–24.
Kawaguchi, Y., 1993. Physiological, morphological, and histochemical characterization of three classes of interneurons in rat neostriatum. J. Neurosci. 13, 4908–4923.
Kirn, J.R., DeVoogd, T.J., 1989. Genesis and death of vocal control neurons during sexual differentiation in the zebra finch. J. Neurosci. 9, 3176–3187.
Lewis, J.W., Ryan, S.M., Arnold, A.P., Butcher, L.L., 1981. Evidence for a catecholaminergic projection to area X in the zebra finch. J. Comp. Neurol. 196, 347–354.
Li, X.C., Jarvis, E.D., Alvarez-Borda, B., Lim, D.A., Nottebohm, F., 2000. A relationship between behavior, neurotrophin expression, and new neuron survival. Proc. Natl. Acad. Sci. U. S. A. 97, 8584–8589.
Lisman, J.E., Zhabotinsky, A.M., 2001. A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly. Neuron 31, 191–201.
Mansuy, I.M., Shenolikar, S., 2006. Protein serine/threonine phosphatases in neuronal plasticity and disorders of learning and memory. Trends Neurosci. 29, 679–686.
Marchand, R., Lajoie, L., 1986. Histogenesis of the striopallidal system in the rat. Neurogenesis of its neurons. Neuroscience 17, 573–590.
Nordeen, E.J., Nordeen, K.W., 1988. Sex and regional differences in the incorporation of neurons born during song learning in zebra finches. J. Neurosci. 8, 2869–2874.
Ouimet, C.C., Miller, P.E., Hemmings Jr., H.C., Walaas, S.I., Greengard, P., 1984. DARPP-32, a dopamine- and adenosine 3′:5′-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. III. Immunocytochemical localization. J. Neurosci. 4, 111–124.
Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N., Ferriero, D.M., 2002. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann. Neurol. 52, 802–813.
Rasika, S., Alvarez-Buylla, A., Nottebohm, F., 1999. BDNF mediates the effects of testosterone on the survival of new neurons in an adult brain. Neuron 22, 53–62.
Reiner, A., Laverghetta, A.V., Meade, C.A., Cuthbertson, S.L., Bottjer, S.W., 2004. An immunohistochemical and pathway tracing study of the striatopallidal organization of area X in the male zebra finch. J. Comp. Neurol. 469, 239–261.
Reiner, A., Perera, M., Paullus, R., Medina, L., 1998. Immunohistochemical localization of DARPP32 in striatal projection neurons and striatal interneurons in pigeons. J. Chem. Neuroanat. 16, 17–33.
Scharff, C., Nottebohm, F., 1991. A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J. Neurosci. 11, 2896–2913.
Scharff, C., White, S.A., 2004. Genetic components of vocal learning. Ann. N.Y. Acad. Sci. 1016, 325–347.
Scott, B.B., Lois, C., 2007. Developmental origin and identity of song system neurons born during vocal learning in songbirds. J. Comp. Neurol. 502, 202–214.
Singh, T.D., Nordeen, E.J., Nordeen, K.W., 2005. Song tutoring triggers CaMKII phosphorylation within a specialized portion of the avian basal ganglia. J. Neurobiol. 65, 179–191.
Soderling, T.R., Derkach, V.A., 2000. Postsynaptic protein phosphorylation and LTP. Trends Neurosci. 23, 75–80.
Soha, J.A., Shimizu, T., Doupe, A.J., 1996. Development of the catecholaminergic innervation of the song system of the male zebra finch. J. Neurobiol. 29, 473–489.
Sohrabji, F., Nordeen, E.J., Nordeen, K.W., 1990. Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. Behav. Neural Biol. 53, 51–63.
Sohrabji, F., Nordeen, E.J., Nordeen, K.W., 1993. Characterization of neurons born and incorporated into a vocal control nucleus during avian song learning. Brain Res. 620, 335–338.
Solis, M.M., Doupe, A.J.S., 1999. Contributions of tutor and bird's own song experience to neural selectivity in the songbird anterior forebrain. J. Neurosci. 19, 4559–4584.
Svenningsson, P., Nishi, A., Fisone, G., Girault, J.A., Nairn, A.C., Greengard, P., 2004. DARPP-32: an integrator of neurotransmission. Annu. Rev. Pharmacol. Toxicol. 44, 269–296.
Williams, H., Mehta, N., 1999. Changes in adult zebra finch song require a forebrain nucleus that is not necessary for song production. J. Neurobiol. 39, 14–28.