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Neurochemical Organization and Experience-Dependent Activation of Estrogen-Associated Circuits in the Songbird Auditory Forebrain

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Abstract

The classic steroid hormone estradiol is rapidly produced by central auditory neurons in the songbird brain and instantaneously modulates auditory coding to enhance the neural and behavioral discrimination of acoustic signals. Although these recent advances highlight novel roles for estradiol in the regulation of central auditory processing, current knowledge on the functional and neurochemical organization of estrogen-associated circuits, as well as the impact of sensory experience in these auditory forebrain networks, remains very limited. Here we show that both estrogen-producing and -sensitive neurons are highly expressed in the caudomedial nidopallium (NCM), the zebra finch analog of the mammalian auditory association cortex, but not other auditory forebrain areas. We further demonstrate that auditory experience primarily engages estrogen-producing, and to a lesser extent, estrogen-responsive neurons in NCM, that these neuronal populations moderately overlap, and that acute episodes of sensory experience do not quantitatively affect these circuits. Finally, we show that whereas estrogen-producing cells are neurochemically heterogeneous, estrogen-sensitive neurons are primarily glutamatergic. These findings reveal the neurochemical and functional organization of estrogen-associated circuits in the auditory forebrain, demonstrate their activation and stability in response to sensory experience in behaving animals, and highlight estrogenic circuits as fundamental components of central networks supporting sensory processing.

Keywords

17 β -estradiol; NCM; estrogen; avian

INTRODUCTION

The steroid hormone 17 β -estradiol (E2) influences the physiology of sensory brain areas and, therefore, shapes the perceptual processing of sensory signals. In the auditory system, in particular, earlier research has shown that hearing thresholds and efficiency are tightly linked to circulating E2 levels in humans and can be manipulated through estrogen-replacement therapy (Davis & Ahroon, 1982; Coleman *et al.*, 1994; Golub *et al.*, 2004; Walpurger *et al.*, 2004). A significant literature also exists demonstrating that women with Ullrich-Turner syndrome, who are deficient in E2, exhibit lower auditory processing

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efficiency and an array of hearing pathologies (Hultcrantz & Sylven, 1997; Gungor *et al.*, 2000).

Little doubt now exists that E2 affects hearing function, and it is currently widely accepted that hormones act at the level of the cochlea. However, exciting evidence from multiple research groups has recently shown that E2 directly regulates central auditory neuronal physiology. This sensory-neuroendocrine interaction has been recurrently studied in the caudomedial nidopallium (NCM), the songbird analog of the mammalian auditory association cortex (reviewed in Maney & Pinaud, 2010). NCM neurons respond vigorously to auditory stimulation in awake animals and are selective for species-specific communication signals (Chew *et al.*, 1996; Velho *et al.*, 2005; Pinaud & Terleph, 2008). NCM is also a site where a significant overlap exists between the auditory and neuroendocrine systems. A large population of NCM neurons expresses aromatase (estrogen-synthase) and each of the estrogen receptors (ERs; ER α and ER β) (Bernard *et al.*, 1999; Metzdorf *et al.*, 1999; Saldanha *et al.*, 2000; Saldanha & Coomaringam, 2005; Tremere *et al.*, 2009). These findings indicate that neurons in NCM, a forebrain area involved in auditory processing, synthesize and are affected by E2 locally.

Consistent with this view, auditory experience was shown to rapidly enhance E2 levels in the NCM of freely-behaving animals (Remage-Healey *et al.*, 2008). Our group showed that this brain-generated E2 increases, in real-time, the gain of auditory-driven responses in NCM neurons via a non-genomic mechanism that involves pre-synaptic suppression of local GABAergic transmission (Tremere *et al.*, 2009). We also recently showed that a main functional role for this E2-modulation is to increase auditory coding efficiency in order to optimize the neural and the behavioral discrimination of acoustic signals in behaving animals (Tremere & Pinaud, 2011). These findings indicate that E2 is a central component of the neural mechanisms supporting sensory processing, and sensory-based behaviors, in the adult vertebrate brain.

Despite significant strides in our understanding of how brain-generated E2 modulates central auditory physiology and behavior, little is known about the organization of estrogen-associated circuits in the auditory forebrain. Here we address this gap by showing that estrogen-producing and -sensitive neurons are heavily and selectively distributed in the adult NCM, and that the density of these neurons is highly stable in response to acute sensory experience. We further show that auditory experience primarily engages estrogen-producing, and to a lesser extent, estrogen-sensitive neurons in NCM, indicating that sensory input may predominantly modulate E2 levels in NCM, relative to the sensitivity of neurons to E2. Finally, we demonstrate that estrogen-associated circuits in NCM are neurochemically heterogeneous.

MATERIAL AND METHODS

Animals, Song Stimulation and Tissue Preparation

We used a total of 21 zebra finches (*Taeniopygia guttata*; n = 11 males and 10 females) that were bred and housed in our animal facility at the University of Oklahoma Health Sciences Center. All animal experimentation protocols were approved by OUHSC's Institutional Animal Care and Use Committee and are in accordance with NIH guidelines. Because no sex differences were detected in any of the parameters analyzed in this work, we have combined data between males and females.

All animals were removed from the flight aviary and kept overnight in individual sound-proof boxes (~76 × 31 × 28 cm), under a 14 h/10 h light/dark cycle (lights on at 6:00 am). The following day, animals were stimulated for 30 min, 1 h or 2 h with playbacks of a

medley of previously unheard conspecific songs (4 novel songs; inter-stimulus interval of 10 s; 70 dB mean sound pressure level, SPL). These groups will be referred to in the remainder of the text as 30 min (n = 6), 1 h (n = 6) and 2 h groups (n = 5). A final group of animals consisted of unstimulated controls (n = 4). This stimulation protocol has been repeatedly shown to markedly induce the mRNA expression of the immediate early gene *zenk*, which reliably reveals song-responsive neurons in NCM (Mello *et al.*, 1992; Mello & Ribeiro, 1998; Mello *et al.*, 2004; Mello & Pinaud, 2006). Given that peak *zenk* mRNA expression is detectable 30 min after stimulation onset (Mello & Clayton, 1994; Mello & Ribeiro, 1998), all studies involving *zenk* expression in the present work have exclusively focused on the 30 min group.

After reaching group criteria, animals were decapitated; brains were rapidly extracted, included in embedding medium (Tissue-Tek; Sakura Finetek, Torrance, CA) and flash-frozen in a dry-ice/ethanol slush. In order to carry out unbiased stereological quantitative analyses in tissue processed for single- and double- fluorescence in-situ hybridization (see below), we conducted systematic-random samples of brain sections collected throughout NCM. Brain sections were cut on a cryostat at 40µm, at the parasagittal plane, were thaw-mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and kept at -80°C until further processing.

Riboprobe Generation for In-Situ Hybridization

We used the QIAprep Spin miniprep kit (QIAGEN Inc., Valencia, CA) to purify pBluescript plasmids containing the zebra finch homologues of: 1) *zenk* (a.k.a., *egr-1*, NGFI-A and *krox-24*), an activity-regulated zinc-finger transcription factor whose expression has been repeatedly and reliably used to map song-responsive neurons in NCM (Mello *et al.*, 1992; Mello & Ribeiro, 1998; Mello *et al.*, 2004); 2) the 65 kDa glutamic acid decarboxylase (GAD65), one of the synthetic enzymes of GABA, and a marker for inhibitory neurons; 3) the vesicular glutamate transporter 2 (vGlut2), a standard marker for excitatory neurons; 4) aromatase (ARO), a marker for estrogen-producing cells; 5) estrogen receptor α (ER α) and 6) ER β both of which are markers for estrogen-sensitive neurons. Clones were originally obtained by screening a λ gt10 cDNA library, through polymerase chain reaction (PCR) cloning or derived from the ESTIMA clone collection, which is publicly available at http://titan.biotech.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=songbird (Mello *et al.*, 1992; Pinaud *et al.*, 2004; Pinaud *et al.*, 2007; Replogle *et al.*, 2008).

Plasmids were linearized or excised with adequate restriction enzymes and purified using QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Sense and antisense riboprobes were generated through in-vitro transcription, following protocols that we have developed and described in detail previously (Pinaud *et al.*, 2008a; Tremere *et al.*, 2009; Pinaud & Jeong, 2010). In brief, riboprobes were labeled with a digoxigenin (DIG) nucleotide labeling mix, which contains DIG-labeled uridine triphosphate (UTP; Roche Diagnostics Corp.), and were subsequently purified in Sephadex G-50 columns. We used 1 ng/µl of purified probe per brain section (added to the 16 µl of hybridization solution, which consisted of 50% formamide, 2 × sodium phosphate, sodium chloride and EDTA (SSPE), 1 µg/µl bovine serum albumin (BSA), 1 µg/µl poly A, 2 µg/µl tRNA in diethyl pyrocarbonate (DEPC)-treated water).

Fluorescence In-Situ Hybridization (FISH)

We have developed, successfully used, and described in detail our FISH protocols previously (Pinaud *et al.*, 2008a; Tremere *et al.*, 2009; Jeong *et al.*, 2010; Pinaud & Jeong, 2010). Briefly, in preparation for FISH, we fixed brain sections in a 3% paraformaldehyde solution in 0.1M phosphate buffer saline (PBS; 5 min). We next washed them in 0.1M PBS

and dehydrated sections by passing them through a standard alcohol series. Brain sections were subsequently incubated in an acetylation solution, which consisted of 1.35% triethanolamine and 0.25% acetic anhydride in water, and were rinsed three times in $2 \times$ SSPE. Sections were again dehydrated in alcohols, allowed to air-dry and were incubated in hybridization solution containing our probe(s) of interest, as detailed above. We next coverslipped slides, sealed them by immersion in a mineral oil bath, and incubated sections overnight at 65°C. The next day, slides were rinsed in chloroform, decoverslipped in $2 \times$ SSPE and were washed in $2 \times$ SSPE (1 h at room temperature), 1.5 h in $2 \times$ SSPE + 50% formamide, and twice in $0.1 \times$ SSPE (at 65°C for 30 min each wash). To inactivate endogenous peroxidase, sections were incubated for 10 min in 0.3% hydrogen peroxide in TNT buffer, which consisted of 0.1M Tris-HCl, pH=7.4, 0.05% Triton-X 100 and 5M NaCl in DEPC-treated water. Subsequently, tissue was sequentially washed in TNT buffer (3×10 min), blocked by incubation in TNB buffer (TNT buffer + 2 mg/mL BSA for 30 min) and incubated for 2 h at room temperature in a solution containing a horseradish peroxidase-conjugated anti-DIG antibody (1: 200 in TNB, Roche Diagnostics Corp., Indianapolis, IN, USA). Sections were then washed in TNT buffer (3×10 min) and incubated for 20 min in a solution containing tyramide-conjugated Alexa 488 (1:200 in amplification buffer, provided by manufacturer; Invitrogen, Eugene, OR, USA). Brain sections were washed in TNT buffer (3×5 min), counterstained with Hoechst, washed again in TNT buffer (3×10 min) and coverslipped with Aquamount. In addition to sense strand hybridization controls, we also carried out incubations where the anti-DIG antibody was omitted, to control for the specificity of the labeling.

Double FISH (dFISH)

We previously developed and detailed our dFISH protocol, which was used here to label two mRNAs at single cell resolution (Pinaud *et al.*, 2008a; Jeong *et al.*, 2010; Pinaud & Jeong, 2010). Briefly, we co-hybridized antisense riboprobes of interest (e.g., *zenk* and ER β , or vGlut2 and ARO). One riboprobe was labeled with DIG, as described above for FISH, and the remaining with biotin, using a biotin labeling mix that contained biotin-labeled UTP (Roche Diagnostics Corp.). After hybridization, sections were washed and developed for the biotin-labeled riboprobe, exactly as detailed above for FISH, using an HRP-conjugated anti-biotin antibody (1:100 in TNT buffer, for 2 h at room temperature; Vector Laboratories, Burlingame, CA). Tissue was then incubated in tyramide coupled with Alexa 594 in amplification buffer for 30 min (1:100 at room temperature). Peroxidase inactivation was carried out by incubating sections in 0.3% hydrogen peroxide for 20 min. Subsequently, the second riboprobe was detected by incubating sections in an HRP-conjugated anti-DIG antibody, as above for FISH, and in tyramide coupled to Alexa 488 (1:200 in amplification buffer). Sections were then washed in TNT buffer (3×5 min), counterstained with Hoechst, washed again in TNT buffer (3×5 min) and coverslipped with Aquamount. For all dFISH studies, we also used the reverse combination of tyramide reagents. Importantly, to control for the effectiveness of the peroxidase inactivation between labels, we carried out additional dFISH reactions where we only omitted the anti-DIG antibody, in which case signal was only observed in the appropriate filter. Finally, we ran additional controls where the anti-biotin antibody was omitted to verify the specificity of the biotin labeling (Pinaud *et al.*, 2008a; Pinaud & Jeong, 2010).

Stereological Quantification and Statistical Analysis

We quantified the number of cells labeled by each riboprobe of interest using the optical fractionator method, an unbiased stereological approach, in a single reference space (NCM). The optical fractionator is considered an unbiased method because the cell counts are not affected by shape, size, spatial distribution or orientation of the neurons analyzed, and accounts for potential biases introduced by the brain-slicing procedure itself.

We conducted counting on an Olympus AX-70 microscope equipped with appropriate filters and a motorized stage, that was coupled to a personal computer integrated with Neurolucida software (MicroBrightField). The boundaries of NCM were outlined in each brain section under low power (4 $\times$), and consisted of the ventricle in its dorsal, caudal and ventral aspects, and the boundary with Field L in its rostral aspect. These boundaries can be readily identified with Hoechst staining. Labeled cells were counted at high power (63 $\times$) with a guard volume of 2 μ m, which was included to avoid artifacts on the sliced surface of the brain sections. More specifically, we collected all sections covering the medio-lateral extent of NCM and systematically-uniform-random (SUR) sampled one-third of these sections. We quantified the total numbers of labeled cells per unit area (mm³) by using the following sampling fractions: thickness sampling fraction (height of dissector divided by section thickness), area sampling fraction (area of sampling frame divided by area of the x-y sampling step) and section sampling fraction (number of sections sampled divided by total number of sections). The area of the sampling frame was 4900 μ m², which derived from a sampling square of 70 $\times$ 70 μ m in length and width, respectively. A step size of 185 μ m in the x-axis and 185 μ m in the y-axis was used for all sections. Finally, the height of the dissector was 13 μ m. To pass counting criteria, positive neurons exhibited at least two-thirds of a labeled cytoplasm continuum that was clearly identifiable around the nucleus, unlabeled cell nucleus and clearly labeled nucleolus (the latter visualized with Hoechst counter-staining).

We calculated the percentages of cells labeled for each riboprobe relative to the total number of neurons per area based on Hoechst counter-staining. When detected under the adequate filter, neurons labeled with Hoechst display lightly, non-homogeneously stained nuclei and conspicuous nucleoli, whereas glial cells usually display strongly, homogeneously stained nuclei. Neurons can be readily identified with this counterstaining even within clusters. Notably, our neuronal numerical densities obtained with Hoechst were not significantly different from those obtained with Nissl-stained sections. Values obtained for each animal were averaged within groups and compared using a standard analysis of variance (ANOVA) and Tukey post-hoc tests, with an alpha value set at 0.05.

Imaging and Photomicrography

Images included in the figure plates were taken with either an Olympus AX-70 or a Nikon TE2000-E epifluorescence microscope coupled to a Nikon Photometrics Cool Snap ES digital camera. High power images were obtained with a Leica SP2 MP confocal microscope. Microscopes were connected to desktop computers equipped with Metamorph software, which was used for image acquisition. Adobe Photoshop software was used to assemble the final image plates.

RESULTS

To study the anatomical properties of ARO, ER α and ER β , we carried out FISH in brain sections with antisense riboprobes derived from cDNA fragments of each of the genes of interest, as above, using protocols that we previously developed and described in detail (Pinaud *et al.*, 2008a; Tremere *et al.*, 2009; Jeong *et al.*, 2010; Pinaud & Jeong, 2010). We identified ARO-, ER α - and ER β -positive cells in the zebra finch NCM and their anatomical distribution was in close agreement with previous reports, as revealed both by radioactive ISH and FISH, as well as immunocytochemical methods (Shen *et al.*, 1995; Bernard *et al.*, 1999; Metzdorf *et al.*, 1999; Saldanha *et al.*, 2000; Saldanha & Coomaringam, 2005; Pinaud *et al.*, 2006; Tremere *et al.*, 2009). An unbiased quantification of these neuronal groups has not been previously conducted, and it is unknown the extent to which each of these cell groups account for the overall neuronal population in NCM. To address these issues, we used a stereological approach to quantify the data from our FISH studies, which

provide clear neuronal identification with single-cell resolution (Pinaud *et al.*, 2008a; Tremere *et al.*, 2009; Pinaud & Jeong, 2010).

ARO and ER β -Positive Cells, but not ER α -Positive Neurons, are Highly Prevalent in NCM

ARO-positive cells were highly abundant, were primarily concentrated in a “crescent-shaped” domain in the caudal NCM, and this density decreased towards rostral aspects of this auditory area. These findings are in close accordance with previous anatomical studies (Metzdorf *et al.*, 1999; Saldanha *et al.*, 2000; Saldanha & Coomaringam, 2005; Pinaud *et al.*, 2006; Tremere *et al.*, 2009; Maney & Pinaud, 2010). Quantification of our data revealed that, in average, each cubic millimeter (mm^3) of NCM encompassed $126.2 \pm 11.1 \times 10^3$ neurons (mean $\pm$ S.D.). We also determined that NCM contained a mean of $85.6 \pm 11.7 \times 10^3$ ARO-positive neurons per mm^3 , which account for $67.8\% \pm 12.3$ of the overall neuronal population (Fig. 1).

A very low density of ER α -positive cells was consistently identified in neurons within a restricted area in the caudal-dorsal NCM. With the exception of this small area, the vast majority of NCM was devoid of ER α -expressing neurons, consistent with previous reports (Metzdorf *et al.*, 1999; Saldanha & Coomaringam, 2005; Tremere *et al.*, 2009; Maney & Pinaud, 2010). Quantification of our data confirmed that ER α -positive neurons are found in very small numbers in NCM. Specifically, NCM contains $8.76 \pm 1.7 \times 10^3$ neurons/ mm^3 that are positive for ER α , which approximate $6.94\% \pm 1.8$ of NCM's neuronal population (Fig. 1). Due to very low expression of ER α in NCM, no further analyses were conducted with this estrogen receptor in the present study.

In contrast to the ER α results detailed above, we found that ER β -positive cells are abundant and homogeneously distributed throughout NCM, consistent with previous anatomical descriptions (Bernard *et al.*, 1999; Tremere *et al.*, 2009). Quantification of ER β -positive neurons in NCM revealed that these cells were highly numerous ($57.74 \pm 8.7 \times 10^3$ neurons/ mm^3) and accounted for a robust fraction ($45.7\% \pm 8.4$) of the overall population of neurons in this auditory area (Fig. 1).

Quantitatively, the neuronal populations that were positive for ARO, ER α or ER β in NCM were significantly different from each other ($F(2, 9) = 20.9$, $p = 0.0004$; Fig. 1). Importantly, no cells labeled for ARO, ER α or ER β were detected in other auditory areas of the caudomedial forebrain, including the thalamo-recipient field L2 and the caudomedial mesopallium (CMM), suggesting that estrogen-associated circuits are exclusively associated with NCM. Furthermore, sense strand hybridizations did not reveal cells that were positive for these markers (not shown).

In summary, these findings provide unbiased quantitative assessments of ARO-, ER α - and ER β -positive neurons, as well as their ratios of the overall neuronal population in NCM.

Estrogen-Producing and Estrogen-Sensitive Neurons Overlap in NCM

Previous studies in NCM revealed that the degree of overlap between ARO- and ER α -positive neurons is negligible (Saldanha & Coomaringam, 2005), presumably due to the low expression levels of this receptor subtype. The extent to which ARO and ER β are co-regulated in NCM neurons, however, is unknown. We quantitatively address this issue by combining double-FISH, which enables the detection of two mRNA types at single cell resolution, and an unbiased stereological approach. We found that $48.02 \pm 4.2 \times 10^3$ neurons/ mm^3 co-express ARO and ER β mRNAs in NCM. Thus, $56.1\% \pm 7.4$ of all ARO-positive cells in NCM co-localize ER β . This fraction also revealed that $83.1\% \pm 6.3$ of all ER β -positive neurons express ARO mRNA. In sum, estrogen-producing, as revealed by

ARO expression, and estrogen-sensitive circuits, as revealed by ER β , overlap in NCM to a moderate degree.

Estrogen-Associated Circuits are Directly Activated by Birdsong Auditory Stimulation in Freely-Behaving Animals

We next tested the extent to which estrogen-producing (ARO) and estrogen-sensitive (ER β) neurons are engaged by auditory experience. To this end, we used song-induced expression of the immediate early gene *zenk* to detect song-responsive neurons (for reviews, see Mello *et al.*, 2004; Mello & Pinaud, 2006). More specifically, we used dFISH to identify the co-expression of *zenk* and ARO or ER β mRNAs in the NCM of birds stimulated with 30 min of a medley of conspecific songs, a time-point that coincides with peak *zenk* mRNA accumulation (Mello & Ribeiro, 1998; Pinaud *et al.*, 2004; Velho *et al.*, 2005).

In our preparations we found that $122.79 \pm 9.2 \times 10^3$ neurons/mm³ express *zenk* mRNA as a result of 30 min of conspecific song stimulation, which reflect $97.3\% \pm 4.6$ of the overall neuronal population in NCM. These findings are highly consistent with previous assessments of the NCM's neuronal population that exhibit a *zenk* response (Mello *et al.*, 1992; Mello & Ribeiro, 1998; Mello & Pinaud, 2006).

The dFISH results showed high co-localization of cells expressing the mRNAs for *zenk* and ARO, which reveals song-responsive, estrogen-producing neurons (Fig. 2A–C). Quantitative analysis revealed that $82.14 \pm 6.2 \times 10^3$ neurons/mm³ expressed both markers. These data showed that $66.9\% \pm 7.3$ of the overall population of song-responsive neurons in NCM, as revealed by *zenk* expression, co-localize ARO. Importantly, $96.0\% \pm 4.6$ of all ARO-positive neurons in NCM co-express *zenk*, suggesting that auditory experience engages a robust population of neurons that potentially produce estrogens in this auditory area.

Curiously, a smaller population of cells co-expressed *zenk* and ER β mRNAs (Fig. 2D–F). Specifically, quantification of our dFISH data revealed that $39.17 \pm 2.8 \times 10^3$ neurons/mm³ were positive for both markers. Considering the overall *zenk*-expressing population in NCM, $31.9\% \pm 8.1$ co-expressed ER β . Notably, when considering all ER β -positive neurons, we found that $67.8\% \pm 6.4$ co-expressed *zenk* mRNA. Finally, we identified song-responsive (*zenk*-positive) neurons that were ARO- or ER β -negative. Although the specific nature of these phenotypes is unclear, they could reflect neurons activated by auditory experience that do not belong to estrogen-associated networks, cells that target direct components of estrogen-related circuitry in NCM (e.g., through efferent projections) or neurons that express alternative estrogen receptors (e.g., ERX or GPR30; Figs. 2C and F, arrowheads). Similarly, we detected neurons that were positive for ARO or ER β , but negative for *zenk*, which reflect neurons that failed to be stimulated by the acoustic parameters of the song stimuli used in our studies, cells that are not song-responsive or neurons that do not express *zenk* (Figs. 2C and F, arrows). Control (unstimulated) animals exhibited negligible numbers of *zenk*-positive neurons in NCM and, therefore, were not quantified.

In summary, these findings show that birdsong auditory stimulation in freely-behaving songbirds engages primarily estrogen-producing neurons, and to a lesser extent, estrogen-sensitive cells in NCM.

Auditory Experience Does Not Impact the Population of ARO- and ER β -Positive Neurons in NCM

Auditory experience increases estradiol levels and modulates the long-term physiology of the awake NCM (Remage-Healey *et al.*, 2008; Tremere & Pinaud, 2011). One potential mechanism for these two observations is that sensory drive influences the recruitment and/or transcriptional activity of estrogen-producing and -sensitive networks in NCM, respectively.

As an initial effort to address this issue, we quantified and compared the density of ARO and ER β -positive neurons in animals that were exposed to 30 min, 1 h or 2 h of conspecific song stimulation, as well as in unstimulated controls, which were kept in silence overnight. We found that the numerical densities of ARO-positive cells were not significantly different across experimental groups: 86.47 ± 17.6 , 85.82 ± 17.3 , 87.11 ± 17.4 and $83.93 \pm 17.3 \times 10^3$ cells/mm³, for control, 30 min, 1 h and 2 h groups, respectively; $F(3, 12) = 0.01$; $p = 0.99$). Likewise sensory experience did not affect the population of ER β -positive neurons: 58.33 ± 17.4 , 56.72 ± 17.3 , 58.89 ± 17.3 and $57.79 \pm 17.3 \times 10^3$ cells/mm³, for control, 30 min, 1 h and 2 h groups, respectively; $F(3, 12) = 0.002$; $p = 1.0$). These findings indicate that estrogen-associated circuits in NCM are highly stable in response to acute auditory experience.

Estrogen-Associated Circuits are Neurochemically Heterogeneous in NCM

Next, to gain insight into the neurochemical identity of estrogen-producing and estrogen-sensitive neurons in NCM, we carried out dFISH studies with the zebra finch homologues of the vesicular glutamate transporter 2 (vGlut2), a reliable marker for excitatory neurons, and the 65 kDa glutamic acid decarboxylase (GAD65), a standard marker for inhibitory neurons (Fig. 3).

We found that ARO-positive neurons were neurochemically heterogeneous. Quantification of sections processed for dFISH revealed that $58.9\% \pm 6.3$ of ARO-positive cells were excitatory, as they co-localized with vGlut2, with the remaining 41% presumably consisting of inhibitory cells (Fig. 3A–C; Fig. 4). These observations were congruent with our findings that $36.9\% \pm 5.2$ of ARO-positive neurons co-expressed GAD65 (Fig. 3D–F; Fig. 4). Even though ER β -positive cells were also neurochemically heterogeneous, this neuronal population was largely biased towards a primarily excitatory network. In particular, our dFISH results showed that $73.8\% \pm 7.4$ of ER β -positive cells were excitatory, based on the co-localization with vGlut2 (Fig. 3G–I; Fig. 4). The remaining 26%, presumably composed of GABAergic cells, was quantitatively well aligned with our results obtained with the GAD65 probe, which revealed that $31.1\% \pm 5.9$ of ER β -positive cells co-localized with GAD65 mRNA (Fig. 3J–L; Fig. 4).

In summary, these studies revealed that estrogen-producing and estrogen-sensitive circuits in NCM are neurochemically heterogeneous, and are composed of both excitatory and inhibitory neurons. Although the population of ARO-positive cells is relatively balanced between glutamatergic and GABAergic neurons, neurons that express ER β are primarily excitatory.

DISCUSSION

Others and we have recently provided clear evidence that brain-generated E2 directly regulates central auditory processing in the adult songbird brain, and consequently shapes auditory-based behaviors in freely-behaving animals (Maney *et al.*, 2006; Remage-Healey *et al.*, 2008; Vyas *et al.*, 2008; Svec & Wade, 2009; Tremere *et al.*, 2009; Vyas *et al.*, 2009; Remage-Healey *et al.*, 2010; Sanford *et al.*, 2010; Tremere & Pinaud, 2011). Even though these recent advances markedly highlighted the extent to which a neurohormone shapes sensory processing, and causally implicated brain-generated E2 as a key modulator of central auditory physiology, our current understanding of the functional and neurochemical organization of estrogen-associated circuits in the songbird auditory forebrain remains very limited. Our work provided a first effort to address this gap in our knowledge. We showed that the NCM of adult zebra finches exhibits a large density of neurons that produce and respond to estrogens (ARO- and ER-positive, respectively), consistent with earlier reports (Shen *et al.*, 1995; Bernard *et al.*, 1999; Metzdorf *et al.*, 1999; Saldanha *et al.*, 2000; Saldanha & Coomaringam, 2005; Pinaud *et al.*, 2006; Tremere *et al.*, 2009). Our results,

however, provided unbiased quantitative analyses of ARO, ER α and ER β mRNA expression at single-cell resolution, their ratios of the overall neuronal populations in NCM, and quantified the degree of overlap of ARO and ER β -positive neurons. Moreover, we have demonstrated that although these neuronal populations, are directly activated by birdsong auditory stimulation, sensory experience predominantly engages estrogen-producing cells, and to a lesser degree, estrogen-responsive neurons.

Interestingly, we found that acute episodes of sensory input do not affect the density of either cell group, suggesting that estrogen-associated circuits in NCM are highly stable in response to auditory experience. Finally, our findings showed that estrogen-producing neurons in NCM are composed almost equally of inhibitory and excitatory neurons, and that estrogen-responsive cells exhibit a dominantly excitatory phenotype. These efforts provide the first quantitative assessment of the effect of sensory experience on estrogen-associated networks and on the neurochemical organization of these circuits.

Estrogen-Associated Networks are Prevalent and Engaged by Experience in NCM

By using unbiased stereological methods, we found that nearly 68% of the overall neuronal population of NCM is composed of ARO-positive cells, suggesting that nearly two-thirds of neurons in this central auditory area are putatively involved in the production of estrogens. Furthermore, a large fraction of neurons in NCM are potentially sensitive to local estrogen levels. Both estrogen receptors are clearly expressed in NCM. Cells expressing ER α are highly scarce, accounting for only 7% of NCM's neuronal population, but were consistently detectable in the caudal-dorsal NCM. In contrast, ER β is the dominant receptor subtype by far; nearly half of all NCM neurons express ER β and are distributed homogeneously throughout this central auditory area. Our observations are in good agreement with previous reports on the distribution of ARO and estrogen receptors in the songbird NCM (Shen *et al.*, 1995; Bernard *et al.*, 1999; Metzdorf *et al.*, 1999; Saldanha *et al.*, 2000; Fusani *et al.*, 2001; Saldanha & Coomaringam, 2005; Pinaud *et al.*, 2006; Tremere *et al.*, 2009). Importantly, our quantitative analyses demonstrated that these cell groups account for a very substantial fraction of the overall neuronal population of NCM, emphasizing the notion that estrogen-based signaling is central to the physiology of a central auditory area.

Our findings also revealed that both estrogen-producing and estrogen-sensitive neurons are engaged by sensory experience in behaving animals, as revealed by zenk mRNA expression. Although most NCM neurons (>95%) exhibit zenk induction in response to playbacks of multiple conspecific songs, the absence of zenk expression does not conclusively reveal neurons that are unresponsive to auditory stimuli. Such neurons could express other activity-regulated genes such as c-fos or arc, as previously shown in NCM (Velho *et al.*, 2005). This possibility, however, would likely reflect a very small population of song-responsive neurons, given that most of these activity-regulated genes are co-regulated in NCM (Velho *et al.*, 2005), and that in our preparations zenk is induced by song in roughly 97% of all neurons. At any rate, considering the population of zenk-positive neurons in NCM, we found that approximately 67% of these cells are ARO-positive, but only 32% expressed ER β . Our findings also revealed that nearly 96% and 68% of ARO- and ER β -positive neurons in NCM are activated by birdsong auditory stimulation, respectively. The neuronal populations expressing ARO and ER α are largely non-overlapping (Saldanha & Coomaringam, 2005), but a significant fraction of ER β -positive neurons co-express ARO. Thus, the robustness of estrogen-associated circuits, their overlap, and engagement by birdsong auditory stimulation in NCM raise the possibility that two non-exclusive models may account for the effects of sensory experience in the physiology of this auditory area. First, auditory stimulation may primarily affect estrogen synthesis and consequently, the population of ARO-positive cells. Second, auditory experience may predominantly engage cells that are positive for estrogen receptors and, thereby, influence the sensitivity of cells to

local levels of estrogens. Our findings favor a model by which auditory experience primarily impacts the production (or levels) of estrogens in NCM and, to a lesser extent, the sensitivity of cells to estrogens. This proposal is also compatible with recent findings suggesting that E2 levels in NCM are markedly and rapidly modulated by auditory experience in freely-behaving animals (Ramage-Healey *et al.*, 2008), and that local inhibition of ARO activity significantly disrupts song-evoked neural responses (Tremere *et al.*, 2009; Ramage-Healey *et al.*, 2010; Tremere & Pinaud, 2011), and the neural (Tremere & Pinaud, 2011) and behavioral discrimination of songs in behaving animals (Ramage-Healey *et al.*, 2010; Tremere & Pinaud, 2011). Although future research is required to segregate the extent to which sensory experience separately, or synergistically, influences the neurophysiological responses of estrogen-producing versus -sensitive neurons, our findings provide valuable information on the “directionality” of the effects of auditory input in estrogen-associated circuits in NCM.

In this study we also tested the impact of acute sensory deprivation and experience in the density of cells that were positive for ARO and ER β . We found that both cell populations are highly stable, being quantitatively unchanged as a result of overnight (~16 h) sound isolation, or up to 2 h of song stimulation. These findings suggest that the transcriptional activities of ARO and ER β are not acutely modulated by experience; thus, the known rises in E2 levels in NCM that occur as a function of sensory input (Ramage-Healey *et al.*, 2008) are likely to involve experience-dependent, post-translational modifications of ARO and/or changes its catalytic activity, rather than alterations in the *de-novo* expression levels of this enzyme. Such a mechanism is congruent with previously reported fast, non-genomic actions of estrogens in the physiology of NCM neurons (Tremere *et al.*, 2009; Ramage-Healey *et al.*, 2010; Tremere & Pinaud, 2011). Moreover, calcium-dependent phosphorylation of ARO has been shown to underlie rapid changes in E2 levels that modify both avian and rodent behaviors, suggesting that hearing-driven rises of E2 in NCM may be mediated through a similar mechanism, in an experience-dependent manner (Foidart *et al.*, 1995; Balthazart & Ball, 2006; Balthazart *et al.*, 2006; Cornil *et al.*, 2006; Taziaux *et al.*, 2007; Balthazart *et al.*, 2009).

Estrogen-Associated Circuits are Neurochemically Diverse

Our findings also demonstrated the neurochemical identity of estrogen-producing and -sensitive neurons in NCM. We found that whereas the population of ARO-positive cells is largely heterogeneous, exhibiting a well-balanced distribution of excitatory and inhibitory neurons, cells that express ER β are heavily biased towards a glutamatergic phenotype. These findings are important, when considered in light of recent research on the neurochemical organization of NCM, because they facilitate the interpretation of observations related to how E2 modulates central auditory neuronal physiology. More specifically, we previously showed that nearly half of the neuronal population of NCM neurons is composed of excitatory, with the remaining half composed of GABAergic, neurons (Pinaud *et al.*, 2004; Pinaud & Mello, 2007; Pinaud *et al.*, 2008b). Furthermore, we previously showed that birdsong auditory stimulation engages a large population of inhibitory neurons (Pinaud *et al.*, 2004), and that GABAergic transmission plays a central role in shaping the auditory coding of songs in the NCM of awake zebra finches (Pinaud *et al.*, 2008b). These earlier findings provide clear evidence that GABAergic cells directly participate in the physiological response to birdsong auditory stimulation in NCM. Taken together with our current findings that most estrogen-sensitive cells in NCM are excitatory, these data suggest that auditory experience may more prevalently influence the population of estrogen-producing cells, as opposed to those neurons that are sensitive to local levels of estrogens.

We also recently showed that E2 plays a central role in regulating neurotransmission in NCM. In particular, we found that E2 selectively suppresses pre-synaptic GABA release

probability onto NCM neurons via a non-genomic mechanism (Tremere *et al.*, 2009). Interestingly, fast glutamatergic neurotransmission is not affected by E2 in NCM (Tremere *et al.*, 2009). These findings indicate that E2 is a main regulator of inhibitory transmission, which is required for shaping the temporal organization of song-evoked responses in NCM (Pinaud *et al.*, 2008b; Tremere *et al.*, 2009; Tremere & Pinaud, 2011). It is, therefore, plausible to suggest that E2 may directly regulate the temporal coding of songs in NCM, via a modulatory influence on local GABAergic transmission. Recent evidence from our group indicates that indeed E2 modulates temporal coding (as well as rate coding) of auditory signals in the NCM of awake animals (Tremere & Pinaud, 2011). Given that the findings presented here demonstrate that GABAergic neurons in NCM are vastly associated with ARO-positive cells, but not ER β -expressing neurons, it is highly likely that a significant fraction of ARO-positive neurons in NCM directly shape in the neural coding of songs. This notion is consistent with a role for E2 in shaping the song-evoked neurophysiological and genomic responses in NCM (Maney *et al.*, 2006; Tremere *et al.*, 2009; Maney & Pinaud, 2010; Remage-Healey *et al.*, 2010; Sanford *et al.*, 2010; Tremere & Pinaud, 2011), and consequently, the neural and behavioral discrimination of auditory signals in awake zebra finches (Remage-Healey *et al.*, 2010; Tremere & Pinaud, 2011). A stringent analysis of how estrogen-producing versus estrogen-sensitive neurons shape the temporal coding of songs in NCM will be a main focus of our future research.

In summary, we provided here a quantitative assessment of the population of estrogen-producing and -responsive neurons in the auditory forebrain of a highly social vertebrate species, demonstrated that these neuronal groups are differentially engaged by behaviorally-relevant communication signals, and are stable following acute epochs of sensory experience. We further demonstrated the neurochemical identity of these neuronal populations. Our findings provide central information on the functional organization of estrogen-associated networks in a central auditory area, and will facilitate the interpretation of past and future findings on the role of brain-generated E2 in neural coding of sensory signals and hearing-based behaviors.

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Figure 1. Estrogen-producing and estrogen-sensitive neurons are highly prevalent in NCM
 Shown are the mean ($\pm$ S.D.) numerical densities of the overall population of neurons, as well as ARO-, ER α -, and ER β -positive neurons in the NCM of adult zebra finches obtained with an unbiased stereological approach (see Methods). Plotted to scale are also the percentages of these cell groups relative to the overall neuronal population in NCM (right-side Y-axis). *** $p < 0.0001$.

Figure 2. Double-FISH for *zenk* and ARO, or *zenk* and ER β reveals song-responsive neurons that produce or are sensitive to estrogens in NCM

(A–C) Photomicrographs depicting representative dFISH signal in the NCM highlighting neurons that co-express *zenk* (A) and ARO (B) mRNAs in the NCM of zebra finches stimulated with conspecific songs. Representative neurons that are positive only for *zenk* (arrowheads) or ARO (arrows), or double-labeled neurons (asterisks), can easily be distinguished in the merged image (C). (D–F) Representative example of dFISH signal depicting neurons that co-express *zenk* (D) and ER β (E) mRNAs in NCM. Cells that are positive only for *zenk* (arrowheads) or ER β mRNAs (arrows) can be identified in the merged image (F), along with neurons that co-localize both mRNAs (asterisks). All images were obtained with confocal microscopy. Scale bar = 25 μ m.

Figure 3. Estrogen-producing and -sensitive neurons are neurochemically heterogeneous in NCM (A–F) Photomicrographs depicting dFISH signal for vGlut2, a marker for excitatory neurons (A) or GAD65, a marker for inhibitory neurons (D), and ARO (B, E) mRNAs in the NCM of adult zebra finches. Representative double-labeled neurons can be seen in the merged images (C, F) with asterisks, along with neurons that are positive only for ARO (arrows), or the neurochemical markers (arrowheads). (G–L) dFISH for vGlut2 (G) or GAD65 (J), and ER β mRNAs (H, K), reveals representative cells that are putatively sensitive to estrogen, and that exhibit either an excitatory or inhibitory phenotype. Representative double-labeled neurons can be identified in the merged images (I, L), along with cells that are only positive for ER β (arrows), or labeled only for either vGlut2 or GAD65 mRNAs (arrowheads). All images were obtained with confocal microscopy. Scale bar = 25 μ m.

Figure 4. Neurochemical identity of estrogen-producing and -sensitive neurons in NCM
Shown is the fraction of estrogen-producing (ARO-positive) and estrogen-sensitive (ER β -positive) neurons in NCM that are putatively excitatory, as revealed by co-expression of vGlut2 mRNA, or inhibitory, as revealed by co-expression of GAD65 mRNA. Note that whereas the population of ARO-positive cells is highly heterogenous, the vast majority of ER β -positive neurons exhibit an excitatory phenotype.