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SYMPOSIUM

Testosterone Treatment Mimics Seasonal Downregulation of Dopamine Innervation in the Auditory System of Female Midshipman Fish

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Synopsis In seasonally breeding vertebrates, hormones coordinate changes in nervous system structure and function to facilitate reproductive readiness and success. Steroid hormones often exert their effects indirectly via regulation of neuromodulators, which in turn can coordinate the modulation of sensory input with appropriate motor output. Female plainfin midshipman fish (*Porichthys notatus*) undergo increased peripheral auditory sensitivity in time for the summer breeding season, improving their ability to detect mates, which is regulated by steroid hormones. Reproductive females also show differences in catecholaminergic innervation of auditory circuitry compared with winter, non-reproductive females as measured by tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholaminergic synthesis. Importantly, catecholaminergic input to the inner ear from a dopaminergic-specific forebrain nucleus is decreased in the summer and dopamine inhibits the sensitivity of the inner ear, suggesting that gonadal steroids may alter auditory sensitivity by regulating dopamine innervation. In this study, we gonadectomized non-reproductive females, implanted them with estradiol (E_2) or testosterone (T), and measured TH immunoreactive (TH-ir) fibers in auditory nuclei where catecholaminergic innervation was previously shown to be seasonally plastic. We found that treatment with T, but not E_2, reduced TH-ir innervation in the auditory hindbrain. T-treatment also reduced TH-ir fibers in the forebrain dopaminergic cell group that projects to the inner ear, and likely to the auditory hindbrain. Higher T plasma in the treatment group was correlated with reduced-ir TH terminals in the inner ear. These T-treatment induced changes in TH-ir fibers mimic the seasonal downregulation of dopamine in the midshipman inner ear and provide evidence that steroid hormone regulation of peripheral auditory sensitivity is mediated, in part, by dopamine.

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Introduction

Seasonal-reproducing vertebrates often undergo dramatic structural reorganization of neural networks in the nervous system that can mediate motivational changes in social and reproductive behaviors (Zupanc and Heiligenberg 1989; Nelson et al. 1990). Sensory systems in particular must be adapted to environmental and social conditions which are often unique during the breeding season. The auditory systems of birds, anurans, and fishes undergo seasonal changes in sensitivity and frequency selectivity throughout the brain and periphery (Sisneros and Bass 2003; Goense and Feng 2005; Gall et al. 2013; Caras et al. 2015). These changes can ensure receivers are tuned to environmentally specific spectral properties of mate calls (Sisneros et al. 2004a) and mediate auditory attention and motivated responses to salient reproductive signals (Cousillas et al. 2013; Forlano et al. 2017).

Steroid hormones are critical regulators of seasonal auditory plasticity (Bass et al. 2016). They can induce both structural changes, such as the addition of new sensory receptors, neurons, or synaptic connections (Kurz et al. 1986; Tramontin and Brenowitz 2000; Coffin et al. 2012), and alter the transcriptional profile of cells (Zakon 1998; Rohmann et al. 2013). Although hormonal regulation can occur directly via genomic and non-genomic mechanisms, it can also occur indirectly through intermediary neuromodulators (Caras 2013; Vahaba and Remage-Healey 2018). The neuromodulatory catecholamines, which include dopamine and noradrenaline, are anatomically and functionally conserved across vertebrates (Smeets and González 2000), and have the capability to coordinate neural activity across sensory, motor, and motivational systems (Riters 2012; Ryczko and Dubuc 2017; Schofield and Hurley 2018). As such, hormonal regulation of neuromodulators provides a powerful and flexible mechanism for the integration of environmental cues and internal states to produce appropriate behaviors (Katz and Edwards 2012). Catecholamines have emerged as key mediators of steroid effects on seasonal plasticity, most notably in the central auditory system of song birds (Maney and Rodriguez-Saltos 2016). However, recent studies in the midshipman fish also suggest a role for catecholamines, particularly dopamine, in seasonal plasticity within the peripheral auditory system (Forlano and Sisneros 2016; Perelmuter et al. 2019).

The plainfin midshipman fish (*Porichthys notatus*) is a marine teleost that migrates from deep offshore winter habitats to breeding sites in coastal bays and sounds of northern California and Washington State in the late spring and summer (Hubbs 1920). Males establish territories and nests under rocks in the intertidal zone and produce an advertisement call ("hum") to attract females for breeding (Brantley and Bass 1994). Gravid females are strongly attracted to the hum, showing an unconditioned phonotaxis response to synthesized playbacks of male hums during the breeding season (McKibben and Bass 1998; Zeddies et al. 2010). With the transition from winter non-reproductive to summer reproductive state, females undergo both an increase in peripheral auditory sensitivity at the level of the hair cell receptors and primary auditory afferent neurons that enhances frequency sensitivity, may improve mate detection, and has been linked to regulation by steroid hormones (Sisneros 2009; Forlano et al. 2015b). Plasma estradiol (E_2) and testosterone (T) peak ~1 month prior to the nesting season, when females undergo seasonal gonadal recrudescence (Sisneros et al. 2004b). Importantly, implant experiments that mimic natural levels of E_2 or T induce summer-like primary auditory afferent encoding properties in winter females (Sisneros et al. 2004a). However, it is not fully understood what neural substrates steroids act on to mediate these changes in auditory sensitivity (Forlano et al. 2016).

Catecholaminergic fiber innervation changes seasonally in the auditory system of females, as measured by tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. In the summer, projections are decreased from the dopaminergic periventricular posterior tuberculum (TPp) in the forebrain to the saccule, the main end organ of hearing in midshipman. In contrast, TPp projections to the octavolateralis efferent nucleus (OE), the source of cholinergic efferent projections to the inner ear, are increased in the summer (Fig. 1; Forlano et al. 2014; Forlano et al. 2015a; Perelmuter and Forlano 2017). Catecholaminergic innervation also differs seasonally within auditory divisions of the hindbrain and thalamic forebrain (Fig. 1; Forlano et al. 2015a). Collectively, these shifts in catecholaminergic innervation may in part explain the observed changes in peripheral auditory sensitivity and female attraction to the male advertisement call (Forlano and Sisneros 2016; Forlano et al. 2017; Perelmuter et al. 2019).

We tested the hypothesis that gonadal steroids regulate this seasonal, reproductive state-dependent change in catecholaminergic input to auditory circuitry by manipulating E_2 and T levels in ovariectomized females in winter, non-reproductive condition, and measuring subsequent changes to TH immunoreactive (TH-ir) fibers within anatomically defined nuclei. In addition to the TPp, saccule, and OE, we measured all other nuclei that exhibited seasonal changes in catecholaminergic innervation (Fig. 1): the dorsomedial and dorsolateral divisions of the descending octaval nucleus (DOdm/dl), which receives direct input from the saccular primary auditory afferents; the central posterior nucleus (CP) of the thalamus and the lateral division of nucleus pre-glomerulosus (PGl), both of which have ascending connections and likely send auditory information to the telencephalon (Bass et al. 1994, 2000; Goodson and Bass 2002). Our results support the hypothesis that T initiates region-specific seasonal changes to dopaminergic innervation of the auditory system that may enhance the detection and encoding of social acoustic signals.

Materials and methods

Animals and hormone implant procedure

All experimental procedures performed in this study were approved by the Institute for Animal Care and Use Committees at the University of Washington and CUNY Brooklyn College. Female midshipman fish in non-reproductive condition ($n = 51$) were collected by otter bottom trawl in Puget Sound near Edmonds, WA, between December and January from 2011 to 2016. Females were group housed at 13.5–14.5°C on short day light cycle (9 am to 5 pm) and fed vitamin-enriched frozen shrimp.

Hormone implant procedures described here replicated previously established methods shown to

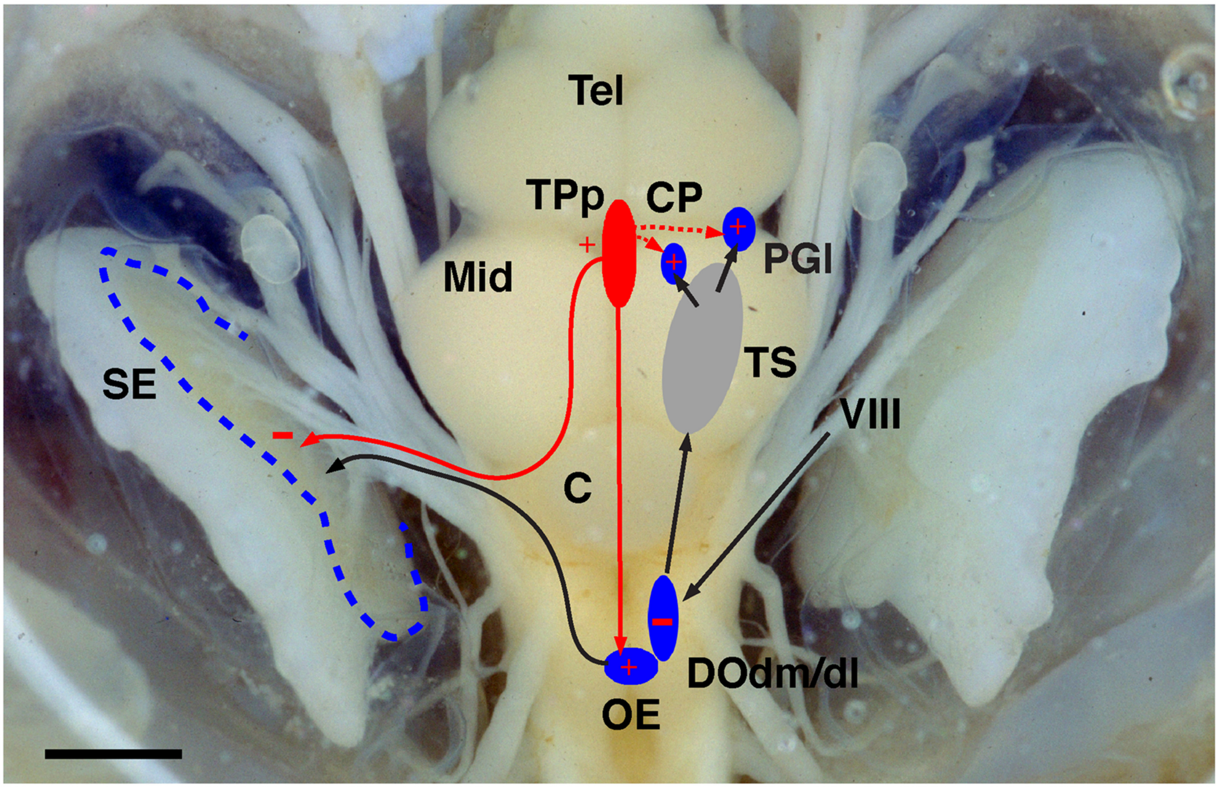

Fig. 1. Regions of the midshipman peripheral and central auditory nervous system that undergo seasonal, reproductive state-dependent changes in TH-ir innervation. Dorsal view of midshipman brain and inner ears. Blue ovals denote approximate location of auditory nuclei and red oval indicates catecholaminergic nuclei investigated in this study. The saccular epithelium (SE, outlined by blue dashed line) contains hair cells and is the principle site of auditory transduction. Primary auditory afferent neurons in the eight nerve (VIII) project to divisions of the hindbrain including the dorsomedial and dorsolateral divisions of the descending octaval nucleus (DOdm/dl). Ascending projections reach the torus semicircularis (TS) in the midbrain, which has projections to the central posterior nucleus of the thalamus (CP) and the lateral division of nucleus preglomerulosus (PGl). The SE receives efferent projections from the cholinergic octavolateralis efferent nucleus (OE) in the hindbrain and from the dopaminergic periventricular posterior tuberculum (TPp,red) in the forebrain. Red pluses indicate regions where TH innervation is higher in the summer (CP, OE, PGl, and TPp). Red minuses indicate regions where TH innervation is lower in the summer (DOdm/dl and SE). Solid arrows indicate connections confirmed with tract tracing, while dashed arrows indicate connections based upon observed fiber projections. Note that SE, VIII, CP, DOdm/dl, PGl, and TS are bilaterally located, although only one hemisphere location is shown. Other abbreviations: C, cerebellum; Mid, midbrain; Tel, telencephalon. Scale bar = 1.5 mm.

induce a summer peripheral auditory encoding phenotype in non-reproductive females (Sisneros et al. 2004a). Prior to implants, females were anesthetized in benzocaine (0.025% in seawater) and their standard length and body mass (g) were taken. They were injected with the analgesic butorphanol (0.04 mg/mL solution) at 1% of body weight. The ventral side of the fish was cleaned with antiseptic solution and a 1 cm incision made just rostral to the anal pore. Ovaries were removed by cauterizing the rostral and caudal ends. Removed ovaries were weighed to calculate gonadosomatic index (GSI; ratio of gonad weight to total body weight). Fish were implanted with uncapped silastic capsules, cut to 1 cm, and filled with either E_2 in coconut oil (1 mg/mL, $n=19$), T in coconut oil (1 mg/mL, $n=11$), or coconut oil alone (control group for E_2, $n=14$; control group for T, $n=10$). The coconut oil/hormone implant mix remains in solid form within the temperature ranges at which the fish were maintained (13.5–14.5°C). The number of capsules was scaled to body mass (1 capsule/13.5 g). Fish were then sutured with polydioxanone clear monofilament absorbable sutures and revived by perfusing fresh seawater over the gills. They were transferred to individual tanks and maintained for 29–31 days.

Tissue collection and steroid hormone measurements

Animals were anesthetized in 0.025% benzocaine dissolved in seawater, the heart was exposed via an incision along the ventral midline, and blood was collected using a heparinized needle for subsequent analysis of circulating steroids. The fish were then transcardially perfused with ice cold teleost ringers, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains and saccules were removed and post-fixed for 1 h, rinsed, and stored in PB with 0.3% sodium azide at 4°C.

Following collection, blood samples were stored at 4°C overnight, centrifuged, and the plasma supernatant was removed and stored at −20°C until use. Plasma E_2 was extracted via double-ether extraction. Supernatants from both extractions were pooled, dried down with N_2 gas, and extract was reconstituted in an equivalent volume of assay buffer. Plasma E_2 concentrations were measured by radioimmunoassay (RIA) as previously described (Sower and Schreck 1982). The intra-assay coefficient of variation (CV) was 2.9% and the inter-assay CV was 13.8%. Plasma T measurements were conducted using an ELISA kit (Cayman, Ann Arbor, MI; #582701) which has been used in numerous other teleost species (Stiver et al. 2015; Teles and Oliveira 2016; Nishiike et al. 2021). To ensure the detection of T in both control and treated fish, we pooled four plasma samples from each group and analyzed them over the following dilution range: 1:1, 1:2, 1:5, 1:10, 1:20, and 1:40. In the T-treated pool, dilutions were within the detectable range of the assay and linear from 1:5 to 1:40 ($r^2=0.99$) and showed acceptable parallelism (slope of pool = −0.98 versus slope of standard curve = −0.74). A dilution of 1:20 resulted in optical density closest to 50%. For the control pool, dilutions were within the detectable range and linear from 1:2 to 1:10 ($r^2=0.98$) and showed parallelism (slope of pool = −0.79 versus slope of standard curve = −0.74). A dilution of 1:5 for controls was chosen as it resulted in optical density closest to 50%. Samples and standards were run in duplicate and imaged on a Synergy 2 plate reader (BioTek Instruments, Winooski, VT). The intra-assay CV was 9.62% and the inter-assay CV was 3.91%. All hormone levels are reported as nanograms per milliliter of plasma (ng/mL).

Immunohistochemistry

Brains and saccules were transferred into 30% sucrose in PB for 24–48 h to cryoprotect the tissue. Using a Leica CM1850 cryostat, brains and saccules were sectioned at 25 and 18 μm (respectively) in the transverse plane, collected onto Superfrost plus slides in two series, and stored at −80°C until processed for immunohistochemistry. Slides were warmed to room temperature, a hydrophobic border was applied using PAP pen (Vector Labs, San Francisco, CA), and processed as follows: 2 × 10 min in 01 M phosphate buffered saline (PBS; pH 7.2), 1 h in blocking solution (10% normal donkey serum & 0.3% Triton-X in PBS) and incubated with primary antibody mixture diluted in blocking solution at room temperature for 16 h. All slides were treated with monoclonal mouse anti-TH (1:1000; MilliporeSigma, Burlington, MA; #MAB318). To visualize cholinergic somata and dendrites in the OE, slides containing this region were double-labeled with polyclonal goat anti-choline acetyltransferase (ChAT; 1:200; MilliporeSigma, #AB144). Slides were then washed 5 × 10 min in PBS + 0.5% donkey serum, incubated for 2 h with secondary antibodies (anti-mouse AlexaFlour 568 and, for OE, anti-goat Alexa 488; Molecular Probes, Eugene, OR) diluted (1:200) in blocking solution, washed 4 × 10 min in PBS and coverslipped with Prolong Gold containing 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Life Technologies, Carlsbad, CA). To distinguish noradrenergic from dopaminergic cells and fibers in brain regions of interest, we examined a set of slides ($N = 5$ animals) from a previous study (Perelmuter and Forlano 2017) that included a rabbit anti-dopamine beta-hydroxylase (DBH) antibody (1:2000; Immunostar, Hudson, WI; #22806) double-labeled with the above TH antibody.

Image acquisition and statistical analysis

All anatomical landmarks, region of interest (ROI) subsampling, and image acquisition methods were based upon our initial study which identified seasonal differences in TH innervation (Forlano et al. 2015a). Slides were coded so microscope operators were blind to condition. Brains were imaged on an Olympus Bx61 epifluorescence compound microscope using MetaMorph image-capture software (Molecular Devices, San Jose, CA). Each brain nucleus was imaged with a 20× objective lens, with exposure time and light level determined for each nucleus and z-stacks consisting of 1 µm steps and 5–7 levels (kept constant within nucleus). Saccular epithelia were imaged on an Olympus Fluoview FV10i confocal microscope with a 60× oil-immersion objective, 2× digital zoom, and acquired as 7 µm z-stacks with a 0.5 µm step size. Brain nuclei (CP, DOdm/dl, OE, PGl, and TPp) and the saccular epithelium were sampled as previously described (Forlano et al. 2015a). There were no statistically significant differences between control and hormone implant groups in the number of images sampled for a given region. All micrographs were post-processed and analyzed using a custom-written ImageJ-based macro system (Timothy and Forlano 2019). Z-stacks were combined into a single projected image using the maximum intensity algorithm. To measure TH-ir fibers in brain micrographs taken on the epifluorescence microscope, the TH-ir channel was background subtracted and signal was automatically thresholded using an average of the Li and Moments methods (Sankur 2004). A border was drawn around the nucleus using DAPI for cytoarchitectural landmarks (CP, DOdm/dl, and PGl) or ChAT-ir to define the somata and dendrites of neurons in the OE. TH innervation of brain nuclei was then quantified as the percentage of TH-ir signal within the ROI, averaged across images (% area). For TPp, percentage TH area was quantified over the entire field of view of the micrograph (433.44 × 330.24 µm). TH-ir neurons in TPp were counted if they displayed a clear nucleus which colocalized with DAPI. To standardize counts, the total number of neurons was divided by the total number of images analyzed per animal. For confocal micrographs from the saccule, the TH-ir signal was automatically thresholded using the Reny Entropy method. A border was drawn around the epithelium, inclusive of hair cells and support cells, where synaptic vesicle-containing TH-ir profiles have been confirmed with electron microscopy (Perelmuter and Forlano 2017). The number and size of TH-ir puncta (0.5–1 µm diameter swellings along TH-ir axons, likely sites for neuromodulator release) were quantified using the Threshold Outline function to distinguish puncta from longitudinal fibers using a size filter of 4–150 pixels and a circularity shape factor of ≥ 0.6 (Forlano et al. 2015a; Timothy and Forlano 2019).

Statistical analyses were carried out using Prism version 7 (GraphPad, San Diego, CA). Independent samples *t*-tests were used to compare hormone levels and TH-ir measurements between treatment groups. Data were visually inspected to assess normality of the residuals. Welch's *t*-test was used when variance between groups was unequal. We report effect size as Cohen's *d*. Individual Pearson's correlations were used to examine the relationship between hormone levels and TH-ir measurements for each ROI, as well as between GSI and TH-ir. Since the evaluation of specific nuclei was determined *a priori* based on seasonal differences, we made no corrections for multiple comparisons. A P-value of <0.05 is reported as statistically significant. Statistics are reported in the text and tables as mean ± standard deviation (SD).

Results

E_2 treatment resulted in circulating levels (3.36 ± 2.28 ng/mL, mean ± SD) that were significantly higher than controls (0.03 ± 0.03 ng/mL, $P < 0.0001$, $d = 2.1$; Fig. 2A). Treated females had E_2 levels within the physiological range measured during the spring pre-nesting season during which reproductive recrudescence begins, while controls had levels comparable to non-reproductive females caught in the winter (Sisneros et al. 2004b). However, TH-ir fiber innervation did not differ between E_2 treated and control groups in CP, DOdm/dl, OE, PGl, nor TH-ir % area or cell number in TPp (Table 1A), all brain nuclei previously shown to exhibit seasonal differences in catecholaminergic input (Forlano et al. 2015a). Similarly, no differences in TH puncta number or size in the saccule were found between treatment groups (Table 1A). Because the range of E_2 levels in the treatment group (0.03–9.82 ng/mL) overlapped at the low end with those in the control group (0.00–0.09 ng/mL), we

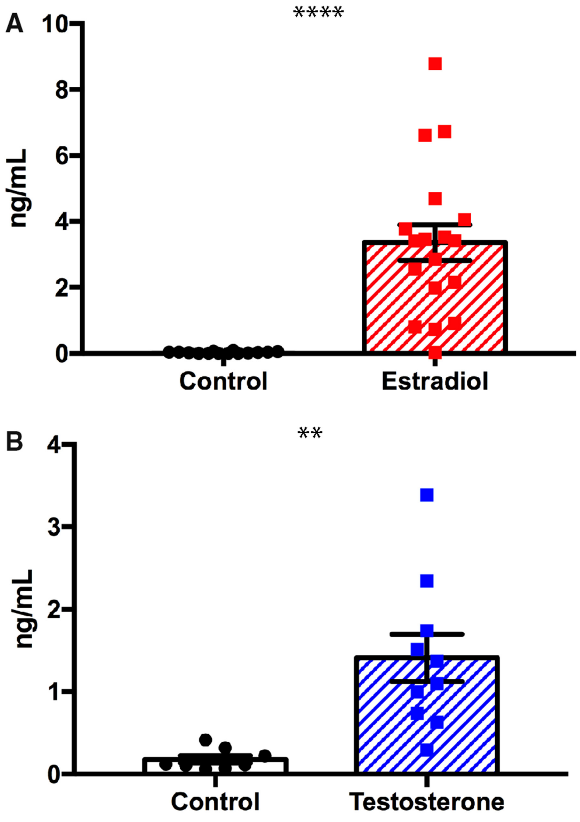

Fig. 2. Steroid levels are elevated after hormone implants. Plasma concentrations of E_2 (**A**) and T (**B**) are significantly higher in treated non-reproductive females relative to controls. $^{**}P < 0.01$, $^{****}P < 0.0001$.

considered that the variability of circulating E_2 within the treatment group could explain the lack of differences in TH innervation. However, there were no statistically significant correlations between plasma E_2 and TH-ir measurements in brain nuclei and saccule (Table 1B). Although all females in this study were acquired in the winter (December to January) when fish are in non-reproductive condition, GSI in the E_2 experiment ranged from 0.53% to 15.92%, overlapping at the upper range with the GSI of females during the pre-nesting period, when gonadal recrudescence begins. However, there was no relationship between GSI and TH-ir measurements across all animals or within the control group (Table 1B).

Since E_2 had no effect, we next tested the hypothesis that T directly regulates seasonal changes in TH. Hormone treated females had circulating levels of T (1.41 ± 0.91 ng/mL) that were significantly elevated compared with controls (0.18 ± 0.13 ng/mL, $P = 0.0017$, $d = 1.79$; Fig. 2B). Treated females had T levels comparable to wild-caught females during the spring pre-nesting season, while controls had levels comparable to non-reproductive fish (Sisneros et al. 2004b). T-treated females had reduced TH-ir % area in TPp (2.62 ± 0.88% versus controls: 3.94 ± 1.3%, $P = 0.046$, $d = 1.19$) (Fig. 3A–E). T-treated and control females did not have a statistically significant difference in the number TH-ir neurons in TPp (T: 13.86 ± 2.6, control: 15.69 ± 3.81, $t(12) = 1.05$, $P = 0.313$, $d = 0.79$) (Fig. 3F). In DOdm/dl T-treated females had lower TH-ir fiber % area (T: 2.47 ± 0.25%, control: 3.21 ± 0.36%, $P = 0.0007$, $d = 2.4$) (Fig. 4A–C). TH-ir fiber area did not differ in CP, OE, or PGl (Table 2A). Although TPp is known to be dopaminergic and presumed to project to DOdm/dl (Forlano et al. 2014), we looked for the colocalization of DBH and TH to exclude the possibility that DOdm/dl also receives noradrenergic input. Locus coeruleus (LC) neurons were TH-ir and DBH-ir positive, TPp neurons were only TH-ir positive and DOdm/dl was devoid of DBH-ir (Fig. 4D–F).

There were no significant differences in TH-ir puncta number or size in the saccule. As with the E_2 experiment, the range of circulating T in the treatment group (0.29–3.39 ng/mL) overlapped with those in the control group (0.07–0.41 ng/mL). We found no relationship between circulating T and TH-ir puncta size (Table 2B), but there was a significant inverse relationship between T levels and TH-ir puncta number in the saccule of the T treated females ($r = -0.88$, $P = 0.01$) (Fig. 5A–C). There were no correlations between circulating T and any brain nuclei (Table 2B). GSI of females in the T experiment varied from 0.78% to 11.38%, overlapping at the upper range with GSI of pre-nesting season females (Sisneros et al. 2004b). There was a significant negative correlation between GSI and TH-ir puncta number within the control group ($r = -0.89$, $P = 0.007$) (Fig. 5D). There was no relationship between GSI and saccular TH-ir puncta area, or TH-ir fiber innervation in brain nuclei (Table 2B).

Discussion

The goal of this study was to determine if the seasonal plasticity of catecholaminergic innervation in the auditory system of female plainfin midshipman fish is under the regulation of circulating gonadal steroid hormones. Unexpectedly, we only found effects from T but not E_2 manipulations. Treatment of non-reproductive, ovariectomized females with T reduced TH-ir innervation in DOdm/dl (Fig. 4A–C and Table 2A), a division of the auditory hindbrain that receives direct input from the saccule (Bass et al. 1994, 2000). Similarly, there is a strong inverse relationship between circulating T levels and TH-ir innervation in the saccule

Table 1 Effect of E_2 treatment on TH-ir in the brain and inner ear

(A) Comparison of TH-ir measurements between E_2-treated and control females

Brain region (TH-ir % area)	Control (mean ± SD)	E_2 treated (mean ± SD)	*t* (df)	*P*-value	Cohen's *d*
CP	2.45 ± 0.43	2.26 ± 0.51	0.96 (22)	0.349	0.40
DOdm/dl	2.42 ± 0.63	2.68 ± 0.77	0.84 (22)	0.41	0.35
OE somata	5.36 ± 1.42	5.24 ± 2.1	0.15 (22)	0.883	0.06
OE dendrites	8.13 ± 2.3	7.65 ± 2.74	0.44 (22)	0.665	0.18
PGl	2.03 ± 0.43	1.91 ± 0.52	0.59 (21)	0.562	0.25
TPp	3.02 ± 0.42	2.89 ± 0.87	0.41 (22)	0.683	0.17
Saccule puncta number	56.71 ± 16.43	57.62 ± 14.1	0.177 (14)	0.909	0.06
Saccule puncta size	0.47 ± 0.02	0.5 ± 0.05	1.402 (14)	0.183	0.72

(B) Correlations of TH-ir measurements with plasma E_2 and GSI

	TH-ir % area versus E_2-levels			TH-ir % area versus GSI					
	E_2 treated			Control			Control & E_2		
Brain region	*r*	*n*	*P*-value	r	*n*	*P*-value	*r*	*n*	*P*-value
CP	0.02	15	0.942	00.53	9	0.143	0.07	24	0.745
DOdm/dl	−0.31	15	0.261	-0.39	9	0.301	−0.20	24	0.354
OE somata	0.04	15	0.874	00.43	9	0.251	0.38	24	0.067
OE dendrites	0.21	15	0.454	00.57	9	0.112	0.31	24	0.139
PGl	0.29	15	0.315	00.59	9	0.091	0.35	23	0.101
TPp	−0.35	15	0.205	−0.19	9	0.624	−0.34	24	0.108
Saccule puncta number	0.27	10	0.443	−0.07	6	0.878	0.14	16	0.591
Saccule puncta size	−0.39	10	0.297	0.2	6	0.669	0.32	16	0.208

(Fig. 5A–C and Table 2B). These effects parallel the lower TH-ir innervation found in the saccule and auditory hindbrain of reproductive summer females relative to winter, non-reproductive fish (Forlano et al. 2015a) (Table 3). Interestingly, T treatment also reduced TH-ir area in the TPp (Fig. 3A–E), whereas in the summer, TH-ir area was higher in this nucleus (Forlano et al. 2015a). Since the number of TH-ir neurons did not vary (Fig. 3F), this difference can be attributed to reduced density of proximal processes from TH-ir TPp neurons (see Fig. 3B and D). We also did not observe any of the summer increases to TH-ir innervation within other auditory nuclei: CP, PGl, and OE (Table 2A). Tract tracing experiments have confirmed that the dopaminergic TPp is the sole source of catecholaminergic input to both the saccule and OE, a cholinergic hindbrain nucleus with efferent projections to the saccule (Forlano et al. 2014; Perelmuter and Forlano 2017). Ascending and local TPp projections within the diencephalon innervate CP and PGl while a robust TH-ir fiber track descends into the hindbrain and appears to innervate DOdm/dl (Forlano et al. 2014). We show here that TH-ir fibers in DOdm/dl are devoid of DBH-ir, ruling out noradrenaline as a neuromodulator in this nucleus (Fig. 4D–F). Since TPp is considered the predominant source of descending dopaminergic innervation in teleosts (Yamamoto and Vernier 2011), it is likely that it is the origin of most if not all TH-ir fibers in DOdm/dl. Taken together, this suggests that T downregulates a specific subpopulation of dopaminergic TPp neurons that projects to the saccule and auditory hindbrain. In our previous seasonal study (Forlano et al. 2015a), a summer decrease in this TPp subpopulation could have been masked by increased projections from TPp to CP, PGl, and OE.

While T-treatment resulted in reduced TH-ir in DOdm/dl, and TPp, the effect in the saccule was dependent on elevated circulating T and evident as a decrease in the number of puncta, without an effect on puncta area (Fig. 5 and Table 2B). This was in contrast to a decrease in both area and number of puncta in summer vs. winter females (Forlano et al. 2015a). Since T-levels ramp up during the Spring, but mating does not occur until the early Summer,

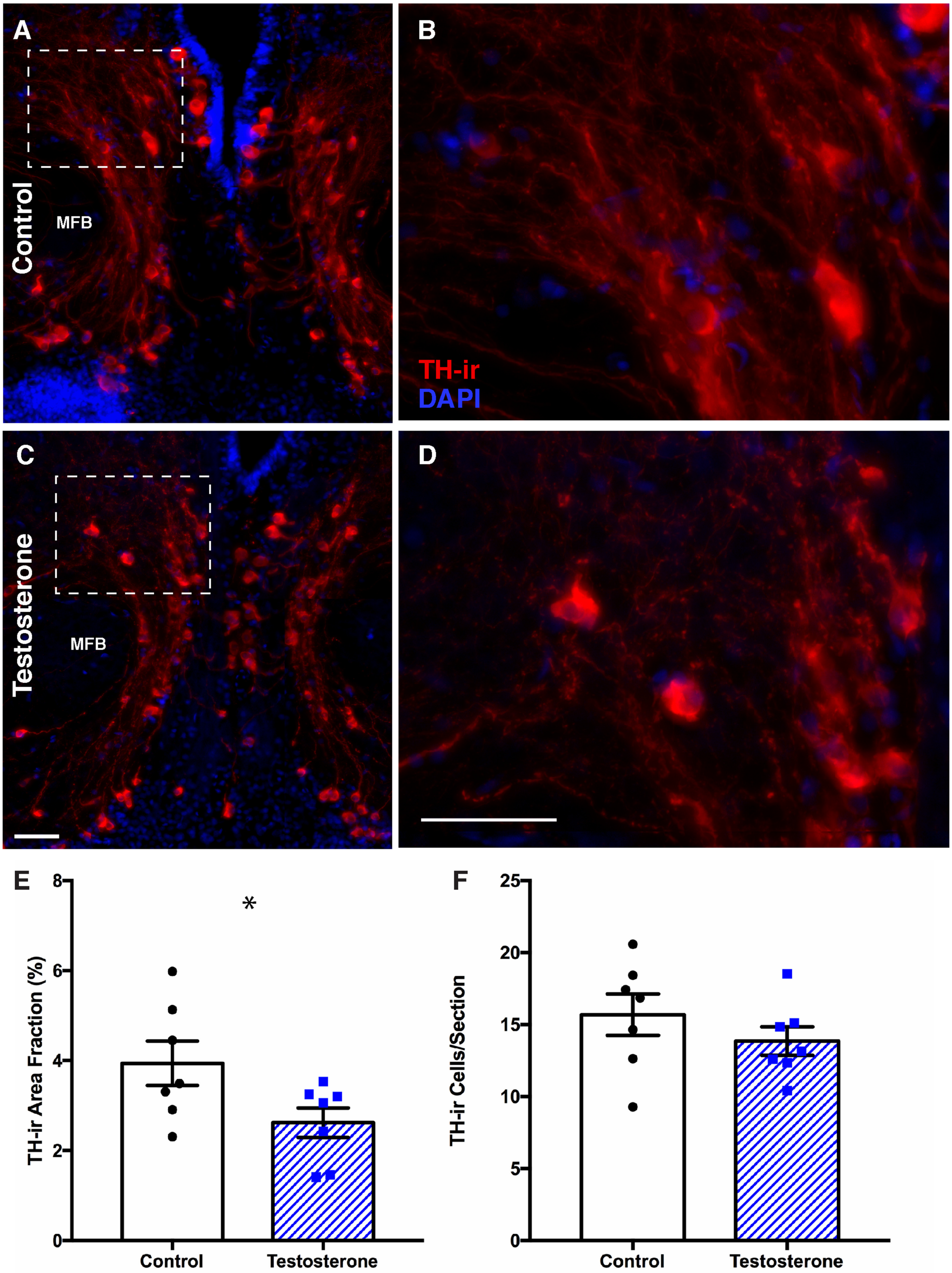

Fig. 3. T reduces TH-ir area in the TPp. Representative transverse sections through the dopaminergic TPp from control (**A**) and T-treated females (**C**). Boxes in (**A**) and (**C**) correspond to higher magnification images of fibers in (**B**) and (**D**), respectively. TH-ir signal (red) is evident in the large, pear-shaped neurons and thick fibers that enter the medial forebrain bundle (label on one side only). T-treated females have significantly lower TH-ir area (**E**). Since there is no statistical difference in the number of TH-ir neurons between groups (**F**), the TH-ir area difference appears to be attributable to reduced proximal fibers from TPp neurons (compare (**B**) and (**D**)). $^*P < 0.05$. Scale bars = 50 µm.

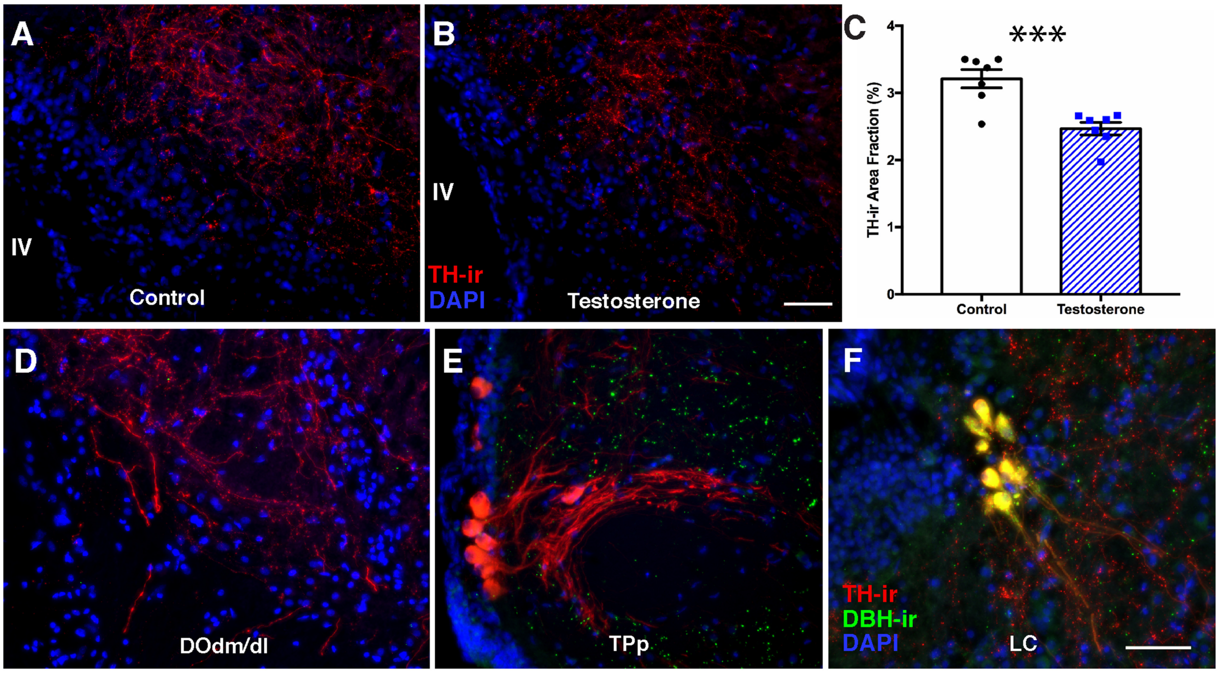

Fig. 4. T reduces TH-ir area in the auditory hindbrain. Representative transverse sections through the DOdm/dl from control (**A**) and T-treated females (**B**) show dense field of TH-ir (red) fibers and terminals dorsolateral to the fourth ventricle (IV). Similar to TPp, T-treated females have reduced TH-ir fiber innervation in DOdm/dl relative to controls (**C**). Double-labeling with TH and DBH (green) shows that TH-ir fibers in DOdm/dl (**D**) and cells and processes in TPp (**E**) are devoid of DBH-ir, whereas neurons in LC are both TH-ir and DBH-ir (**F**). $^{***}P < 0.001$. Scale bars = 50 µm.

1 month following T-treatment may not be sufficient time to induce the full summer phenotype of reduced saccular TH innervation. Indeed, if downregulation of TH begins centrally within TPp, changes in the hindbrain would be expected to precede peripheral changes in the inner ear. A clear difference between T-implant and control groups in the saccule was likely further obscured by an inverse relationship between the number of TH-ir puncta and pre-ovariectomy gonadal state in the control group. Although caught between December and January, some fish had a GSI within the range of pre-nesting females, when the gonads begin to recrudesce (Sisneros et al. 2004b). Our plasma hormone samples, taken ∼30 days after the fish were ovariectomized, would not reflect circulating levels when the animals were first captured, but fish with larger GSI may have already begun the transition to a summer phenotype, including reduced TH-ir puncta in the saccule. Fish caught later in the winter (i.e., January) would have had even more time for increasing levels of T to affect the plasticity of dopaminergic efferents.

Androgens are known regulators of catecholamines across vertebrate taxa. In rodents, T can both increase or decrease the number of dopaminergic cells and fibers, depending on brain region (Simpkins et al. 1983; King et al. 2000; Johnson et al. 2010). Treatment with T increases catecholaminergic innervation of song control nuclei in female canaries (Appeltants et al. 2003), but decreases noradrenergic innervation of the auditory midbrain of male white-throated sparrows (Matragrano et al. 2013). Methyltestosterone treatment decreases TH mRNA in whole brain of female and male catfish, *Clarius batrachus* (Mamta et al. 2014). Acute and chronic treatment with androgens reduces dopamine synthesis in the pituitary of rainbow trout, but increases TH in the olfactory bulb and preoptic area of the European eel (Hernandez-Rauda and Aldegunde 2002; Weltzien et al. 2006). Thus, effects of T vary considerably by species, sex, and anatomical location. To our knowledge, this is the first reported evidence for steroidogenic regulation of dopamine in the inner ear and auditory brainstem in any vertebrate and perhaps the only species where a comparison has been made between natural seasonal variation and steroid manipulations of catecholamine innervation in the auditory system.

Mechanisms of hormonal regulation of catecholamines

Androgen and estrogen receptors, along with aromatase have been confirmed in the TPp (Forlano et al.

Table 2 Effect of T treatment on TH-ir in the brain and inner ear

(A) Comparison of TH-ir measurements between T-treated and control females

Brain nucleus (TH-ir % area)	Control (mean ± SD)	T treated (mean ± SD)	*t* (df)	*P*-value	Cohen's *d*
CP	3.03 ± 0.8	3.43 ± 1.65	0.56 (11)	0.584	0.31
DOdm/dl	**3.21 ± 0.36**	**2.47 ± 0.25**	**4.49 (12)**	**0.0007**	**2.4**
OE somata	3.15 ± 0.83	3.67 ± 1.05	1.02 (12)	0.327	0.55
OE dendrites	3.62 ± 1.06	4.68 ± 1.53	1.51 (12)	0.157	0.81
PGl	3.2 ± 1.51	3.07 ± 0.9	0.19 (12)	0.849	0.1
TPp	**3.94 ± 1.3**	**2.62 ± 0.88**	**2.22 (12)**	**0.046**	**1.19**
Saccule puncta number	61.67 ± 12.25	61.69 ± 15.13	0.00 (12)	0.998	0.00
Saccule puncta size	0.73 ± 0.07	0.63 ± 0.16	1.55 (12)	0.147	0.83

(B) Correlations of TH-ir measurements with plasma T and GSI

	TH-ir % area versus T-levels			TH-ir % area versus GSI					
	T treated			Control			Control and T		
Brain region	*r*	*n*	*P*-value	*r*	*n*	*P*-value	*r*	*n*	*P*-value
CP	0.25	6	0.638	00.07	7	0.874	0.23	13	0.448
DOdm/dl	0.11	7	0.815	00.00	7	0.998	0.07	14	0.805
OE somata	−0.33	7	0.476	−0.07	7	0.874	−0.21	14	0.469
OE dendrites	0.45	7	0.316	00.36	7	0.431	−0.09	14	0.752
PGl	0.66	7	0.109	−0.05	7	0.922	0.02	14	0.942
TPp	0.41	7	0.359	0.11	7	0.81	0.15	14	0.614
Saccule puncta number	**−0.88**	**7**	**0.01**	**−0.89**	**7**	**0.007**	**−0.57**	**14**	**0.033**
Saccule puncta size	−0.39	7	0.297	0.34	7	0.459	0.04	14	0.888

Bold measurements indicate statistically significant differences ($P < 0.05$).

Table 3 Comparison of seasonal and T induced changes to TH-ir fibers

Region of nervous system	Summer (versus winter)[a]	T treatment (versus control)
CP nucleus of the thalamus	Increases	–
DO nucleus	Decreases	Decreases
OE nucleus	Increases	–
Lateral division of nucleus PGl	Increases	–
TPp	Increases	Decreases
Saccule (SE) – puncta area	Decreases	–
Saccule (SE) – puncta number	Decreases	Decreases[b]

[a] Forlano et al. (2015a)

[b] Lower puncta number correlated with higher T-levels.

2005, 2010; Fergus and Bass 2013), but not yet colocalized with markers for dopamine. It is possible that the reduction of TH-ir innervation we report here could be mediated, in part, by aromatization of T. However, brain aromatase mRNA is reduced from forebrain to hindbrain, specifically in the preoptic area, periaqueductal gray and vocal motor nucleus of midshipman females in the winter, non-reproductive condition compared to pre-nesting spring, when circulating steroids are highest (Forlano and Bass 2005b). This suggests that aromatase is likewise reduced in TPp during the winter, although we cannot preclude the possibility that our steroid treatments altered aromatase expression or neurosteroid synthesis (Forlano and Bass 2005a) . Importantly, we did not see any effect of E_2 in our study, suggesting a direct androgenic action on TPp neurons. An androgen-specific mechanism could be confirmed by co-administering T-implants with an aromatase inhibitor such as fadrozole or using a non-aromatizable androgen such as 5α-dihydrotestosterone or 11-ketotestosterone, although females do not have detectable levels of non-aromatizable androgens (Sisneros et al. 2004b). An androgen response element was identified in the TH promoter of mammals (Jeong et al. 2006) supporting a conserved role for androgenic regulation of TH gene

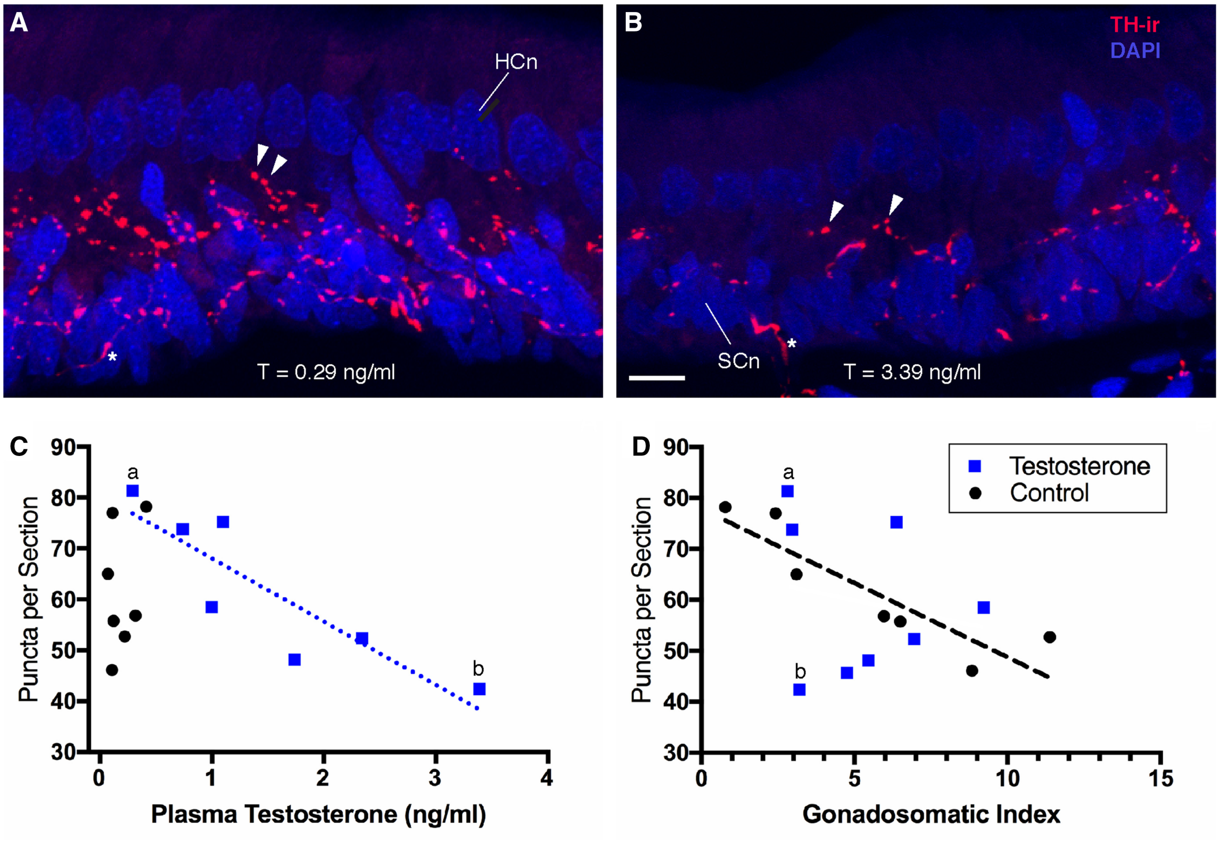

Fig. 5. Plasma T is negatively correlated with TH-ir puncta number. Representative micrographs from T-implanted females showing TH-ir puncta (red, examples labeled with white arrowheads) in a female with the lowest (**A**) and highest (**B**) circulating T-level measured at the time of sacrifice. White asterisks indicate examples of fibers of passage that are excluded from the puncta analysis. Nuclei of hair cells and support cells are labeled with DAPI (blue). Number of puncta per section correlates with plasma T in the implant (blue squares), but not the control group (black circles), $r^2 = 0.76$, $P = 0.01$ (**C**). The variability of puncta number in the control group is explained by reproductive state prior to gonadectomy in the control group, $r^2 = 0.80$, $P = 0.007$ (**D**). Scale bar = 10 µm.

transcription. Since E_2 and T treatment did not alter TH innervation in CP, PGl, and OE, the summer increase of innervation in these nuclei may require both T and E_2 acting synergistically, or these changes may be regulated by another hormone, such as thyroid hormone (Chaube and Joy 2003), 7α-Hydroxypregnenolone (Haraguchi et al. 2015), or melatonin (Chaube and Joy 2002). Melatonin release in fishes is influenced by photoperiod and temperature (Falcón et al. 2007), both of which are potential environmental cues that could initiate gonadal recrudescence in seasonal breeders. Intriguingly, treatment with melatonin increases TH synthesis in the European eel (Sébert et al. 2008) and melatonin receptors are found in midshipman TPp (Feng et al. 2019). The profile of hormonal receptors within dopaminergic TPp neurons is likely to be heterogeneous. Indeed, single-cell RNA sequencing of mammalian hypothalamus has revealed that neuronal subpopulations previously thought to be homogenous based on the expression of a single neuromodulator or neuropeptide, can be further subcategorized based upon unique expression patterns of multiple hormone and peptide receptors (Moffitt et al. 2018). We predict that a subset of dopaminergic TPp neurons will express androgen receptors and project to the saccule and auditory hindbrain, while another population of TPp neurons, projecting to the CP, PGl, and OE, may express other hormone receptors such as melatonin.

Functional implications of T regulation of dopamine

Dopamine reduces the sensitivity of saccular hair cells and the reduction of dopaminergic innervation in the saccule of summer, reproductive females coincides with downregulation of D2a receptors, contributing to a seasonal increase in saccular auditory sensitivity (Perelmuter et al. 2019). T (or E_2) may also regulate the seasonal expression of D2a receptors in hair cells, as has been proposed for calcium-activated potassium and other ion channels

(Rohmann et al. 2013; Fergus et al. 2015). The T-mediated reduction of TH innervation in DOdm/dl could similarly produce a release of dopaminergic inhibition, increasing the sensitivity of auditory hindbrain neurons to advertisement calls. Dopamine inhibits spiking of neurons in the mouse cochlear nucleus (Bender et al. 2010) and reduces background firing rates of primary auditory afferent neurons, enhancing signal-to-noise ratios in a context dependent manner at synapses onto the Mauthner cell in zebrafish (Mu et al. 2012). Electrophysiological studies of seasonal or dopamine-dependent effects may reveal a role for dopaminergic modulation of social acoustic signals in the midshipman auditory hindbrain.

In conclusion, these data support the hypothesis that a pre-spawning increase in T initiates a seasonal, structural change of dopaminergic innervation at the earliest stages of auditory processing. This plasticity likely enhances the ability of females to find and evaluate mates. Androgenic regulation of dopamine may be a broad mechanism by which other seasonally breeding vertebrates synchronize reproductive readiness with auditory system plasticity to optimize reproductive success.

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