Report

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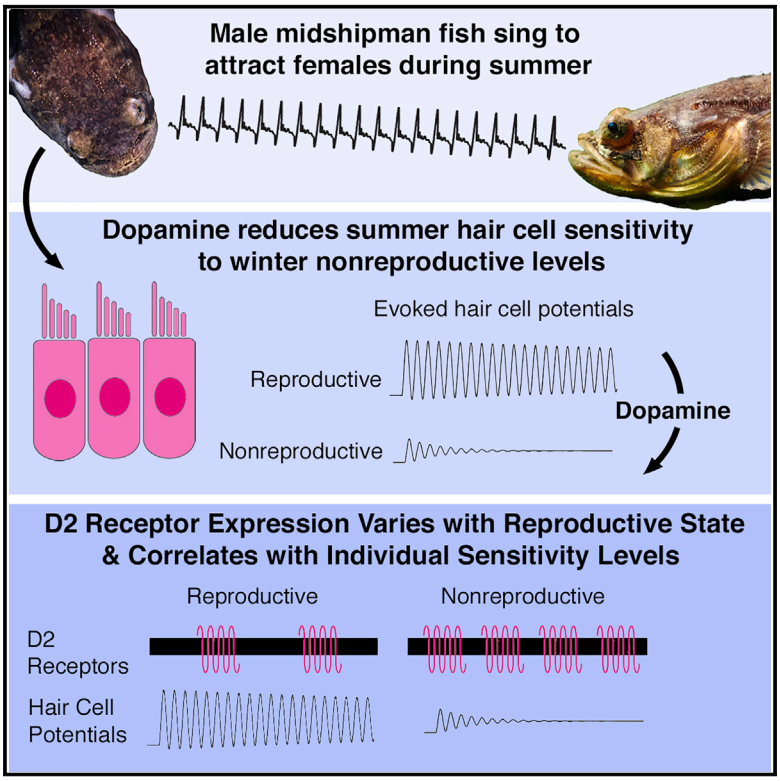
Forebrain Dopamine System Regulates Inner Ear Auditory Sensitivity to Socially Relevant Acoustic Signals

Graphical Abstract



Highlights

- Dopamine reduces inner ear sensitivity in a vocal fish, matching seasonal changes
- Reduced sensitivity is mediated by D2 receptors (D2Rs) expressed in hair cells
- D2 receptors vary with reproductive state and inversely correlate with sensitivity
- Results suggest a role for inner ear dopamine in social-acoustic communication

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In Brief

In mammals and fishes, central dopamine neurons project to the inner ear and could affect the encoding of acoustic signals at the earliest stage of processing. Perelmuter et al. provide evidence from a vocal fish that dopamine contributes to a reproductive-state-dependent shift in inner ear sensitivity, enhancing a female's ability to detect mates.

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Forebrain Dopamine System Regulates Inner Ear Auditory Sensitivity to Socially Relevant Acoustic Signals

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SUMMARY

Dopamine is integral to attentional and motivational processes, but studies are largely restricted to the central nervous system. In mammals [1, 2] and fishes [3, 4], central dopaminergic neurons project to the inner ear and could modulate acoustic signals at the earliest stages of processing. Studies in rodents show dopamine inhibits cochlear afferent neurons and protects against noise-induced acoustic injury [5–10]. However, other functions for inner ear dopamine have not been investigated, and the effect of dopamine on peripheral auditory processing in non-mammalians remains unknown [11, 12]. Insights could be gained by studies conducted in the context of intraspecific acoustic communication. We present evidence from a vocal fish linking reproductive-state-dependent changes in auditory sensitivity with seasonal changes in the dopaminergic efferent system in the saccule, their primary organ of hearing. Plainfin midshipman (*Porichthys notatus*) migrate from deep-water winter habitats to the intertidal zone in the summer to breed. Nesting males produce nocturnal vocalizations to attract females [13]. Both sexes undergo seasonal enhancement of hearing sensitivity at the level of the hair cell [14–16], increasing the likelihood of detecting conspecific signals [17, 18]. Importantly, reproductive females concurrently have reduced dopaminergic input to the saccule [19]. Here, we show that dopamine decreases saccule auditory sensitivity via a D2-like receptor. Saccule D2a receptor expression is reduced in the summer and correlates with sensitivity within and across seasons. We propose that reproductive-state-dependent changes to the dopaminergic efferent system provide a release of inhibition in the saccule, enhancing peripheral encoding of social-acoustic signals.

RESULTS

The dopaminergic innervation of the midshipman fish saccule originates from the periventricular posterior tuberculum (TPp) in the forebrain, and is discrete from cholinergic efferents from the hindbrain [4, 20, 21] (Figures 1A and 1B). Dopaminergic puncta in the saccule do not form synapses, suggesting paracrine release and the potential to modulate hair cells, cholinergic efferent, and primary auditory afferent synapses [22]. A previously reported reduction of dopaminergic puncta size and number in the saccules of summer females (Figures 1B and 1C) [19] coincides with enhanced higher-frequency encoding by saccular afferents [23] and greater sensitivity of hair cells [14, 16]. These are changes that could improve the detection of the dominant harmonic content of male courtship vocalizations (Figure 1D), which propagate more readily in the shallow waters of summer breeding sites [18]. When female midshipman fish are in reproductive condition, they exhibit robust phonotaxis to both natural and synthesized playbacks of the male courtship vocalization [24]. Because we were interested in the effect of dopamine on the ability of females to localize and assess male courtship calls, we evaluated females collected from male nests in the summer, when they are in reproductive condition, most likely to respond to males, and their peripheral auditory sensitivity is maximal [25].

Dopamine Decreases Hair Cell Sensitivity in a Dose-Dependent Manner

Because a reduction of dopaminergic input to the saccule was found in summer females, we hypothesized that dopamine would produce an inhibitory effect on the sensitivity of saccular hair cells. We recorded auditory evoked receptor potentials from populations of hair cells in the saccule to evaluate the effect of iontophoretic injection of dopamine on hair cell sensitivity. Consistent with our prediction, iontophoresis of dopamine

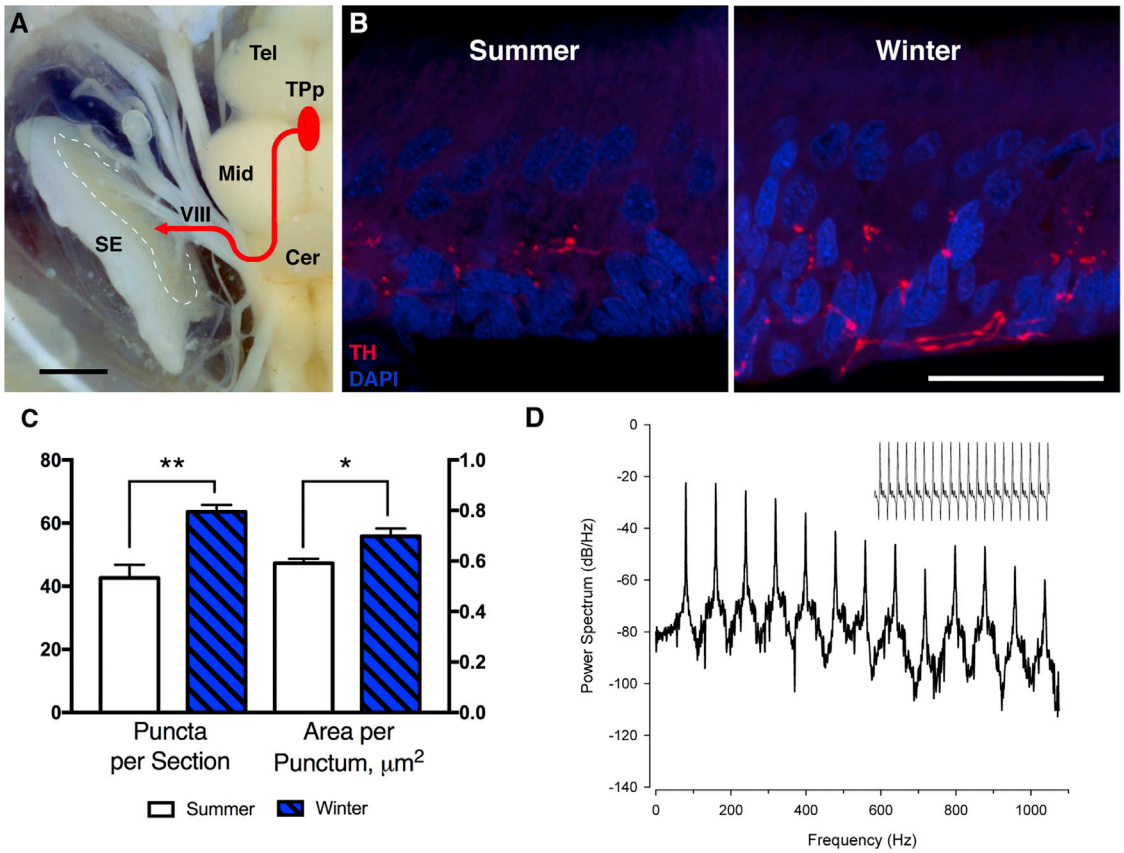

Figure 1. Background: Origin and Seasonal Changes of Dopaminergic Input to the Saccule

(A) Dorsal view of midshipman brain depicting dopaminergic projection (red) from the TPp to the saccular epithelium (SE). Cer, cerebellum; Mid, midbrain; Tel, telencephalon; TPp, periventricular posterior tuberculum; VIII, eighth nerve. Scale bar, 1.5 mm.

(B) Micrographs from summer and winter females showing seasonal change to dopamine (DA) innervation (TH, tyrosine hydroxylase; red) of saccule. Nuclei of hair cells and support cells labeled with DAPI (blue). Scale bar, 25 μm.

(C) Number and size of DA puncta are reduced in summer females.

(B) and (C) were adapted from [19]. Error bars show SEM; *p = 0.017, **p = 0.001.

(D) Power spectrum of male courtship call. Power is nearly equal between fundamental frequency (~100 Hz) and the first 3 harmonics, with significant harmonic peaks up to 1,000 Hz. Waveform of call is shown in the inset at top right (2 s long).

resulted in a dose-dependent increase in auditory thresholds. Both 5 mM ($p < 0.0001$) and 50 mM ($p < 0.0001$) doses of dopamine raised thresholds to pure tones ranging from 75 to 405 Hz, compared to vehicle-injected controls (Figure 2A). In contrast, the effect of a 1 mM dose of dopamine was not significantly different from vehicle ($p = 0.1636$). Because the effect of dopamine was independent of frequency (all p values > 0.05), we averaged the threshold change relative to controls across frequencies for each dose. The 5 mM and 50 mM doses increased auditory thresholds on average by 14.81 and 21.47 dB re 1 μPa, respectively, and were significantly different from one another and the 1 mM dose (Figure 2B; all p values < 0.0001). The dose-dependent decrement in hair cell sensitivity induced by exogenous dopamine is consistent with a physiological effect mediated by receptors (STAR Methods). Further support for a physiological effect is provided by fact that the change induced by 5 mM and 50 mM dopamine in summer, reproductive females resulted in auditory thresholds that were similar to previously published thresholds from unmanipulated winter, non-reproductive females (Figure 2C) [16].

Although most previous midshipman studies evaluated auditory sensitivity between 75 and 425 Hz [14, 15, 17, 23, 26], the power spectrum of the male courtship call contains significant harmonic peaks above 400 Hz. Thresholds are detectable up to 1,025 Hz in reproductive fish [27]. We likewise obtained thresholds up to 1,025 Hz from a majority of fish in both the control (90%) and 1 mM dopamine (80%) conditions (Figures 2A and 2D). We were unable to obtain thresholds above 705 Hz for fish treated with 5 mM dopamine or above 405 Hz for fish treated with 50 mM dopamine (Figures 2A and S2A). The proportion of evoked responses obtained at higher frequencies after 5 mM and 50 mM dopamine treatment was significantly different from controls (Figure S2A; $p = 0.0031$ and 0.0002), whereas the 1 mM dopamine condition was indistinguishable from control ($p = 0.91$). It is possible that treatment with the higher doses of dopamine increased thresholds beyond the range we could test, as our underwater speaker cannot reliably reproduce tones above 155 dB re 1 μPa. Playback experiments evaluating female responses to male hums all employ stimulus intensities that range from 130 to 140 dB re 1 μPa measured at the position of

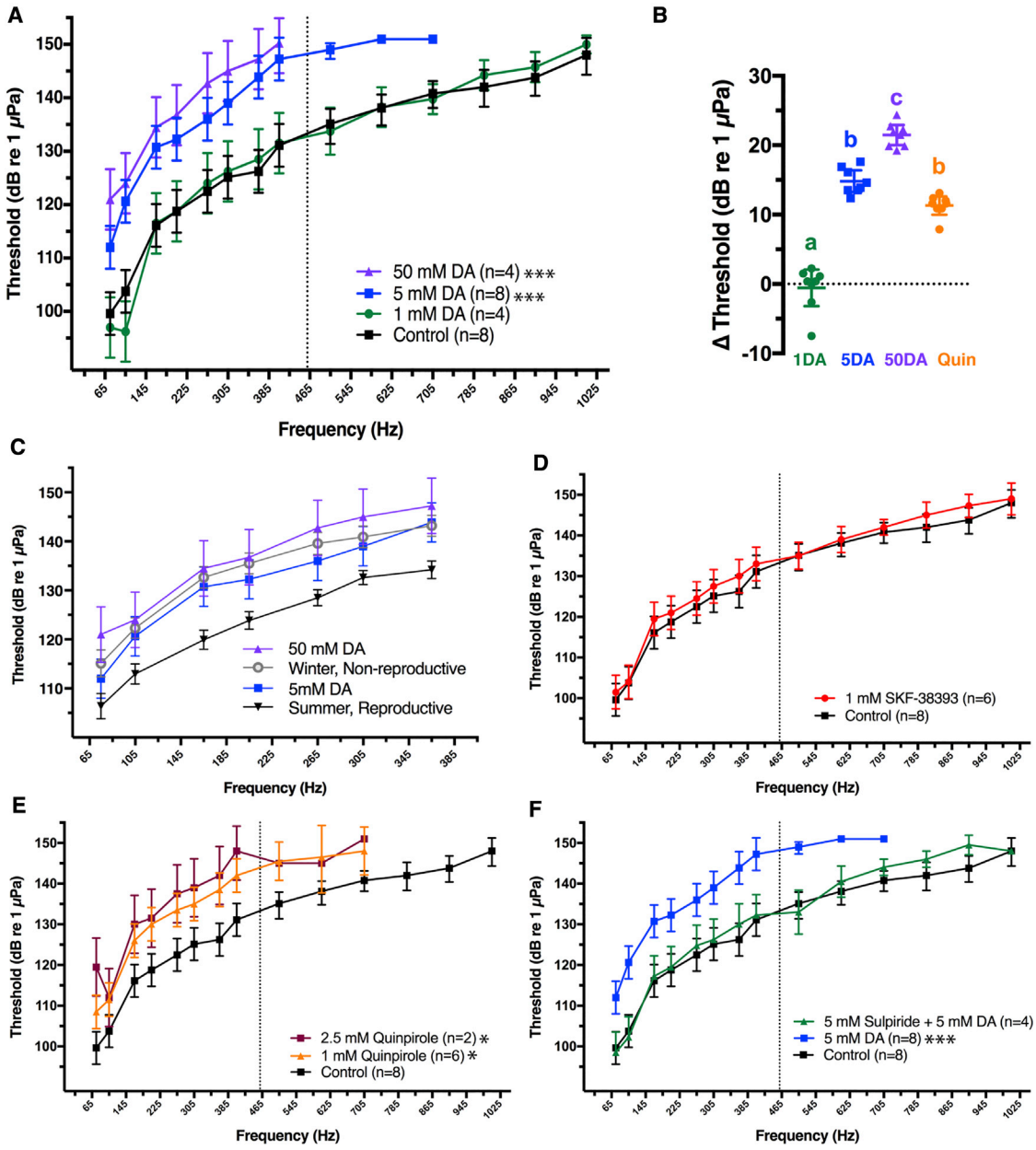

Figure 2. DA Decreases Hair Cell Sensitivity in Summer Females via a D2-like Receptor Mechanism

(A) Threshold tuning curves of hair cells showing that the DA-induced increase in thresholds depends upon dose. The dotted vertical line indicates cutoff frequency above which the incidence of supra-threshold responses was reduced, precluding threshold determination and inclusion of higher frequencies in the statistical model.

(B) The average change in threshold, relative to control, is significantly higher in fish treated with 5 mM DA and 50 mM DA, as compared to 1 mM DA. Quinpirole, a D2R agonist, induces a similar change as 5 mM DA. Because there was no difference in the effect of quinpirole dose (see F), the 2.5 mM and 1 mM doses were combined. Different letters indicate statistically significant differences. All p values < 0.0001.

(C) Summer fish treated with 5 mM and 50 mM DA have thresholds that are similar to winter, non-reproductive fish. Seasonal thresholds were replotted from [16].

(D) The D1-family agonist, SKF-38393, produces no threshold change.

(E) The D2-family agonist, quinpirole, increases thresholds. Both doses produce comparable effects.

(F) Sulpiride, a D2-family antagonist, blocks the change induced by 5 mM DA.

Asterisks indicate treatments that are significantly different from control. *p < 0.01, **p < 0.001, ***p < 0.0001. All error bars represent 95% confidence intervals. See also Figures S1 and S2.

animal release and 86 to 109 cm from the speaker [24, 28, 29], and we have recorded male hums at the entrance of nests as high as 153–161 dB re 1 μPa [30]. Saccular thresholds above 160 dB are unlikely to support detection and recognition of biological relevant stimuli. Thus, the shift of thresholds above this cutoff in the higher-dose dopamine groups has meaningful

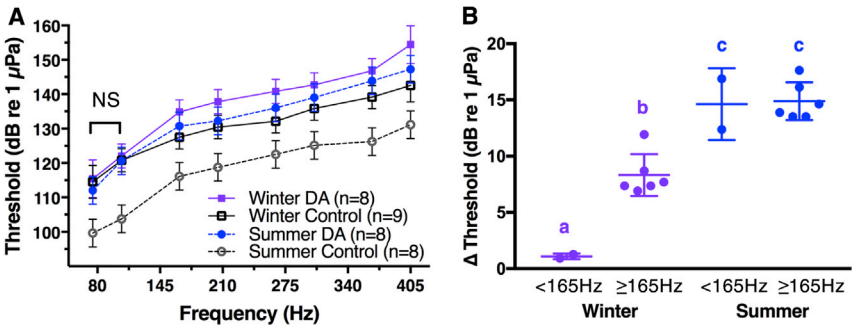

Figure 3. DA Decreases Hair Cell Sensitivity in Winter Females

(A) 5 mM DA significantly increases saccular hair cell thresholds in both summer (reproductive) and winter (non-reproductive) fish. In winter, this effect is frequency dependent, occurring at and above 165 Hz. Error bars represent 95% confidence intervals. NS = no significant difference relative to controls.

(B) Average threshold changes induced by DA for low versus high frequencies in winter and summer fish. Different letters indicate statistically significant differences; all p values < 0.01. Error bars represent SDs.

consequences for the organism, namely a reduced ability to detect and process higher-frequency information, especially as sound pressure decreases by 6 dB with each doubling of distance from the sound source [18].

Dopamine Decreases Hair Cell Sensitivity via a D2-like Receptor

The effects of dopamine are mediated by both D1 (generally excitatory) and D2 (generally inhibitory) receptor families [31]. We next sought to determine which receptor family mediates the auditory threshold change induced by exogenous dopamine. Using the same methods of drug delivery (iontophoresis) and evaluation of thresholds using population-level auditory evoked receptor potentials, we found that a broad D1-family agonist, SKF-38393 (1 mM), produced no difference from control injections (Figure 2D; p = 0.6382). In contrast, a broad D2-family agonist, quinpirole, increased thresholds, independent of frequency, at both 1 mM and 2.5 mM concentrations (Figure 2E; main effects, p = 0.0055 and 0.0022; interaction effects, p = 0.2869 and 0.6369). There was no difference in the effect of quinpirole dose (p = 0.2395). The average auditory threshold change induced by quinpirole, irrespective of dose, was 11.3 dB re 1 μPa (Figure 2B). Co-applying 5 mM dopamine with a D2-family antagonist, sulpiride (5 mM), blocked the inhibitory effect of the exogenous dopamine, yielding no threshold differences from control fish (Figure 2G; p = 0.6104). Quinpirole and dopamine had similar effects on higher-frequency sensitivity, with neither group showing thresholds above 705 Hz (Figures 2A, 2E, and S2B). Quinpirole- and dopamine-treated fish had reduced higher-frequency thresholds that were significantly different from controls (Figure S2B; p = 0.001 and 0.0028), whereas SKF-38393 and dopamine-plus-sulpiride-treated fish were indistinguishable from controls (Figure S2B; p = 0.52 and 0.23). These results indicate that saccular hair cells likely express D2-like receptors.

Dopamine Decreases Hair Cell Sensitivity in Both Winter and Summer Females

To determine whether dopamine affects auditory hair cell sensitivity similarly across reproductive states, we evaluated thresholds after iontophoresis of 5 mM dopamine or vehicle in winter, non-reproductive female fish. As in summer reproductive females, exogenous dopamine increased thresholds (Figure 3A); however, a model including both winter and summer fish showed a significant interaction between season and treatment (p = 0.035). A model with only winter fish revealed a significant interaction between frequency and treatment (p = 0.0075), so we performed post hoc pairwise comparisons at each frequency. Dopamine significantly increased auditory thresholds between 165 and 405 Hz (all p values < 0.001), by 8.32 dB on average, relative to vehicle-treated controls, but not at 75 and 105 Hz (Figure 3). In summer fish, the same 5 mM dose of dopamine increased thresholds by an average of 14.63 dB at 75 and 105 Hz and 14.88 dB between 165 and 405 Hz (Figure 3B). Although the general effect of dopamine across seasons is reduced saccular hair cell sensitivity, these results suggest seasonal changes in dopamine metabolism, receptor expression, or downstream signaling mechanisms.

Dopamine Receptor Subtype Expression in the Saccule

Whereas mammals possess five dopamine receptor subtypes, teleost fishes may possess genes for up to fourteen receptor subtypes as a consequence of genome duplication events [32]. Utilizing transcriptomes of the midshipman saccule [33, 34], we identified transcripts for seven dopamine receptor subtypes (Figure S3). Due to the seasonal differences of dopamine fiber innervation [19] and the effects of dopamine on saccular sensitivity (Figures 2 and 3), we hypothesized that dopamine receptor expression would be seasonally labile. We performed quantitative real-time PCR (qPCR) with saccular epithelia from the same summer and winter female fish used for receptor potential recordings and confirmed expression of all seven receptor subtype transcripts. However, only the D2a receptor was differentially expressed, with significant downregulation in summer reproductive fish (Figure 4A; p = 0.0022).

D2a Transcript Expression Correlates with Hair Cell Sensitivity

We next sought to determine whether D2a receptor levels could, at least in part, account for baseline hair cell sensitivity (Figures 4B and 4C). Across seasons, auditory thresholds were positively related to both frequency (p < 0.0001, r^2 = 0.79) and D2a transcript levels (p = 0.0007, r^2 = 0.21), with no interaction between frequency and transcript levels (p = 0.0719). D2a expression was also positively related to thresholds within both summer (p = 0.0063, r^2 = 0.18) and winter (p = 0.043, r^2 = 0.16) fish, with no interaction between frequency and transcript expression (summer, p = 0.2454; winter, p = 0.727). These results suggest that D2a receptor expression levels causally contribute to baseline auditory sensitivity, although given the moderate r^2 values, other factors are likely to play a role.

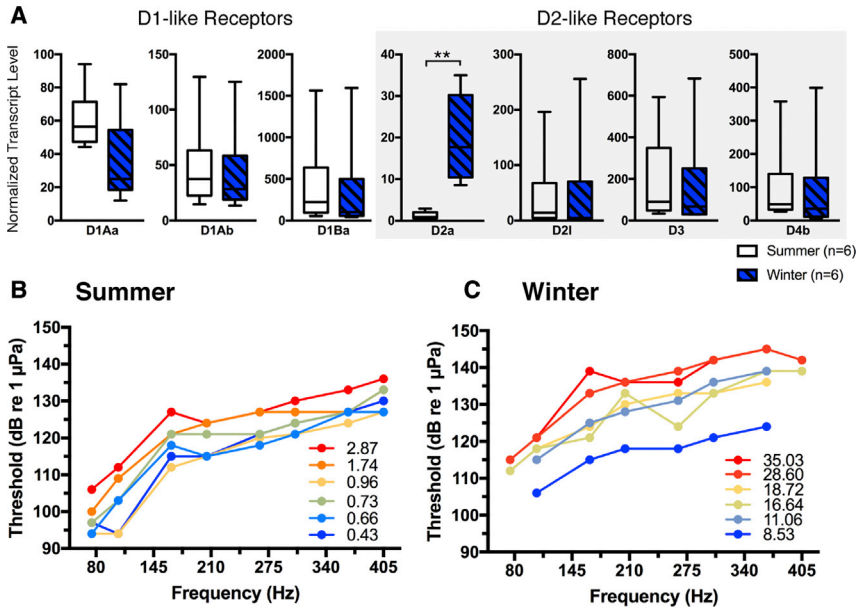

Figure 4. D2a Receptor Expression Varies with Season and Correlates with Threshold

(A) Normalized mRNA expression in saccules from winter and summer fish show that although there are 7 DA receptor subtypes, only the D2a receptor is differentially expressed ($p = 0.0022$). Normalized expression shown as box and whisker plots.

(B and C) Thresholds increase with greater D2a expression across seasons ($p = 0.0007$, $r^2 = 0.21$), and within both summer (B; $p = 0.0063$, $r^2 = 0.18$) and winter (C; $p = 0.043$, $r^2 = 0.16$). Normalized D2a expression levels for each subject are indicated by numbers in the key. The color-coding scheme reflects relative expression levels within a season. See also Figure S3 and Table S1.

DISCUSSION

Mechanisms of Dopamine Inhibition of Hair Cell Sensitivity

D2 receptors could modulate hair cell membrane properties via calcium, potassium, or hyperpolarization-activated cyclic nucleotide-gated (HCN) channels [31]. In support of such mechanisms, trout saccular hair cells express D2 receptors, downstream signaling pathway components (Gαi proteins, adenylyl cyclases), and voltage-gated calcium and HCN channels [35–37]. Intriguingly, large-conductance, calcium-activated potassium (BK) channels, known to be important for seasonal hair cell frequency tuning in midshipman [26], mediate dopaminergic inhibition of nucleus accumbens neurons and gated release of prolactin from lactotrophs in the pituitary [38, 39]. It is tempting to speculate that BK channels in the midshipman saccule could mediate the effects of dopamine we demonstrate here.

Although we show mRNA expression of D1 receptor subtypes in saccular preparations, the lack of an effect of the D1 agonist SKF-38393 on evoked receptor potentials suggests that rather than being expressed in hair cells, these receptors may be localized to support cells or afferent or efferent fibers. Dopamine receptors are localized to primary auditory afferent neurons in mammals [5, 40, 41], and punctate dopamine fibers course through primary afferent ganglia of the midshipman saccule [4] and larval zebrafish lateral line [42]. Ion exchange in cochlear support cells of the guinea pig stria vascularis is inhibited by dopamine [43], suggesting the expression of receptors in these cells. An effect of dopamine on cholinergic efferents cannot be ruled out, considering the close interplay of these neuromodulators in the central nervous system [44]. Larval zebrafish lateral line hair cells express D1Ab receptors and the D1 agonist SKF-38393 increases evoked receptor potentials [3], indicating that the absence of an effect of the D1 agonist in the midshipman saccule is not likely due to drug specificity.

Although dopamine reduced saccular sensitivity in both summer and winter females, the auditory threshold shift in the winter was smaller and frequency specific, only occurring above 105 Hz. Given that dopamine fibers and D2a receptor expression is greater in the winter, one might expect the effect of dopamine to be greater as well. However, because winter baseline thresholds are already dramatically higher than summer, there may be an upper limit to how far sensitivity can be reduced by dopamine. Alternatively, the greater effect of dopamine in summer animals could result from a seasonal reduction of reuptake mechanisms and degradation enzymes. The specific effect of dopamine may also depend on the number and type of ion channels expressed in hair cells, which vary seasonally [34]. A BK channel-specific blocker has larger effects on saccular sensitivity at higher frequencies, whereas a general potassium channel blocker has larger effects at lower frequencies [26]. Therefore, the frequency-dependent effect of dopamine in the winter could result from selective modulation of BK channels, which although expressed at lower levels in the winter [26], could be expressed at a higher ratio relative to other ion channels. Evoked potential thresholds were higher when D2a expression was greatest, both within and across seasons. This suggests a direct role for this receptor subtype in mediating the effects of dopamine and the seasonal changes to saccular sensitivity.

Dopamine Contributes to Adaptive Seasonal Auditory Plasticity

Seasonal saccular plasticity is likely initiated by a pre-migration spike of circulating sex steroids [45] that is causally linked to improved frequency encoding by eighth-nerve saccular afferents [17]. Enhanced frequency sensitivity has been proposed to result from an increased density of hair cells [46] and upregulation of BK channels [26], both of which are correlated with seasonal changes in reproductive-state and steroid hormone levels. Additionally, a transcriptome study identified a suite of candidate genes including several ion channels that are upregulated in summer males [34]. The present study adds centrifugal dopaminergic input as a complementary mechanism for sculpting seasonal frequency sensitivity. Although our study focused on females, we do not expect a sex difference given that males show similar seasonal changes in saccular sensitivity [14, 15].

Further studies will be required to determine whether the summer reduction of dopamine fiber innervation [19] and D2a receptor expression in the saccule are under the regulation of steroid hormones.

Dopamine Modulation across Timescales

Neuromodulators operate across multiple timescales varying by many orders of magnitude [47, 48]. Although we provide evidence linking peripheral dopamine to seasonal shifts in auditory sensitivity, this does not preclude other acute functions for inner ear dopamine. In midshipman, activity of dopaminergic neurons of the TPp (the source of dopamine to the saccule; Figure 1A) is enhanced in males by playbacks of male courtship calls [49, 50] and in females correlates with duration of phonotaxis responses to simulated calls [28]. The TPp has widespread projections throughout the central and peripheral nervous system [4, 22, 42, 51], but if neurons specifically projecting to the saccule are tuned to conspecific signals, transient dopaminergic inhibitory feedback to the inner ear could improve signal detection in noise [52] or enhance the contrast between binaural inputs, improving sound source localization [53]. Alternatively, dopaminergic inhibition could serve as a locomotor corollary discharge mechanism, similar to the cholinergic efferent system, which is engaged in males during calling [54, 55]. However, in larval zebrafish, TPp neurons that project to the lateral line show weak anti-correlated activity with swimming and are tuned to mechanosensory stimuli [56], supporting a role for dopamine as a peripheral sensory gain control mechanism.

Conclusion

Our results are the first demonstration of dopaminergic modulation of the peripheral auditory system in a non-mammalian vertebrate. Prior studies of dopamine in the cochlea of rodents also show an inhibitory effect, but largely focus on protection from noise-induced injury as the proposed function [5–8, 10, 40, 57, 58]; however, this is just one possible function of auditory efferent systems [11, 59]. Kirk and Smith [60] suggested that protection from acoustic trauma is unlikely to be an evolved function of auditory efferent systems because the experimental stimuli required to induce damage have few analogs, in terms of both intensity and duration, in the natural environment. Although some natural sound sources, such as volcanic eruptions or thunder, are of sufficient intensity, it has been argued that such extreme sound environments are "rare and discontinuously distributed in time and space" [61] and therefore unlikely to drive the common evolution of auditory efferent systems found in nearly all vertebrates [11]. Although inner ear dopaminergic efferents may offer protection against anthropogenic noises, their function in natural contexts has remained poorly studied. Using a neuroethological model, we show that seasonal changes to the dopaminergic efferent system provide a release of inhibition, contributing to overall peripheral auditory plasticity in midshipman fish that adaptively enhances acoustic communication during social reproductive behavior. The TPp is one of the most evolutionarily conserved dopamine nuclei in vertebrates and is considered homologous to the A11 cell group in mammals [51, 62]. Although projections to the inner ear have only, to our knowledge, been investigated in fishes, A11 in mice has projections to auditory nuclei in the midbrain and hindbrain [63, 64]. Dopamine may similarly mediate peripheral auditory plasticity in other seasonally breeding vocal species, including anurans [65] and birds [66, 67], and play an important role in the peripheral encoding of social-acoustic signals across vertebrates.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.05.055.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.T.P., J.A.S., and P.M.F.; Methodology, J.T.P., A.B.W., J.A.S., and P.M.F.; Investigation, Formal Analysis, & Writing – Original Draft, J.T.P.; Writing – Review & Editing, J.T.P., A.B.W., J.A.S., and P.M.F.; Resources, A.B.W., J.A.S., and P.M.F.; Funding Acquisition, J.T.P., J.A.S., and P.M.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
(±)-SKF-38393 hydrochloride	Sigma-Aldrich	D047
(S)-(-)-Sulpiride	Sigma-Aldrich	S7771
(-)-Quinpirole hydrochloride	Sigma-Aldrich	Q102
Dopamine hydrochloride	Sigma-Aldrich	H8502
Critical Commercial Assays		
Quick-RNA MicroPrep Kit	Zymo Research	R1050
SuperScript III Reverse Transcriptase	Invitrogen	18080044
Power SYBR Green PCR Master Mix	Applied Biosystems	4367659
Oligonucleotides		
Primers for qPCR, see Table S1	Sigma-Aldrich	N/A
Software and Algorithms		
Geneious v10.1.3	Biomatters	RRID: SRC_010159; https://www.geneious.com
MATLAB v2007a	MathWorks	RRID: SRC_001622; https://www.mathworks.com
Prism 7.0a	Graph-Pad	RRID: SRC_005375; https://www.graphpad.com
R	The R Foundation	RRID: SRC_001905; https://www.r-project.org

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jonathan T. Perelmuter (jperelmuter@gradcenter.cuny.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Female midshipman fish were hand-collected in reproductive condition in the summer (June 2016) from intertidal nesting sites in Brinnon, WA and in non-reproductive condition in the winter (January 2016, 2018) by trawl in the Puget Sound, WA and Monterey Bay, CA. Fish were group housed in saltwater aquaria at the University of Washington in Seattle, WA and used for physiology experiments within 3 weeks of capture. Standard length (SL), body mass (BM) and gonad mass were recorded for all fish. Sex and reproductive condition were confirmed after each experiment by both visual inspection of the ovaries and evaluating gonadosomatic index (GSI), calculated as 100 x gonad mass/(body mass – gonad mass). Females were considered to be in reproductive condition if they had ovaries with large, developed yellow/orange-yolked eggs (~5 mm diameter) and a GSI greater than 10. Nonreproductive females had ovaries with small white eggs (~1 mm diameter) and a GSI less than 10. This study included 36 reproductive females (mean SL = 16.33 ± 1.3 cm SD, mean BM = 56.8 ± 16.65 g SD, and mean GSI = 22.97 ± 10.48 SD) and 11 nonreproductive females (mean SL = 14.78 ± 3.24 cm SD, mean BM = 45.63 ± 29.09 g SD, and mean GSI = 3.3 ± 2.89 SD). All animal care and experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

METHOD DETAILS

Physiology and Pharmacology

Methods for *in vivo* recording of auditory evoked saccular hair cell receptor potentials were based upon previous studies [14–16, 26, 27, 46, 68]. Animals were anesthetized for surgery by immersion in 0.025% ethyl-*p*-am-ionobenzoate dissolved in seawater for approximately 5 min, until opercular movement ceased, followed by an intramuscular injection of cisatracurium besylate for immobilization and 0.25% bupivacaine for analgesia. After exposing bilateral otic capsules, a 3-4 cm hydrophobic dam was erected around the craniotomy to prevent exposure of the inner ear to salt water. Fish were submerged, secured to a head-holder in a 40-cm diameter tank and positioned 10 cm above an underwater speaker (UW-30, Telex Communications). Water was recirculated via a mouthpiece over the gills and tank temperature was maintained between 14 and 15°C. The tank was situated on a vibration-isolation air table inside a sound attenuation chamber.

Dopamine hydrochloride, quinpirole, SKF-38393 and sulpiride (Sigma) were dissolved in artificial endolymph [26, 69] with 0.1% sodium metabisulfite, and delivered into the extracellular space of the saccule via iontophoresis using 30 minute, 0.5 Hz duty cycle,

through glass microelectrodes with a 30-40 μm tip diameter (Figure S1A). Injection currents were 10 nA for dopamine and 50 nA for all other compounds. This injection method was adapted from a previous study [26]. Pharmacological agents were selected based upon comparable behavioral and physiological effects in teleosts and mammals [3, 70–74]. D1 and D2 receptors have been functionally characterized in eel, goldfish and tilapia and show binding affinities for commercially available dopamine receptor agonists and antagonists that are similar to mammals [75–77]. Initial drug concentrations and injection times were determined based upon published pharmacology studies of dopamine in the rodent cochlea [7, 58], and then adjusted to achieve consistent effects based on pilot experiments (Figures S1B and S1C). Doses reflect the concentration of compounds within the injection electrode. As shown previously [26], the effective concentrations at the site of action (i.e., hair cells) are likely to be considerably less, as compounds must travel from the site of injection to their target, resulting in a concentration gradient. The dynamics of this gradient are influenced by the rate of diffusion, which is in turn determined by factors such as tortuosity of the tissue, bulk flow and clearance/uptake mechanisms [78]. Our injection pipette was positioned at the dorsal aspect of the saccule, approximately 2.5 mm away from the hair cells in the epithelium (Figure S1A). The ejection of a compound from a point source (i.e., a pipette) produces a steep concentration gradient that rapidly decreases with distance from the source. This spatial gradient reaches equilibrium and remains stable over time as long as both the flow rate from the pipette and the clearance rate are constant [79]. For dopamine, additional mechanisms such as breakdown (i.e., via MAO and COMT enzymes) and uptake by dopamine active transporter (DAT) are likely to further decrease the working concentration around hair cells, producing an even steeper local concentration gradient [80]. We estimate that for a 5 mM dose of dopamine, the effective concentration at hair cells will range from 2.9 μM to 17.5 nM. These values are comparable to tonic levels of dopamine in the mammalian nervous system, which have been reported to range from 3 μM to 2 nM [81–86].

Following injection of either dopamine, receptor drugs or vehicle (artificial endolymph) into one saccule, evoked potentials were recorded in response to single tones. For fish treated with dopamine agonists (N = 8), after the D1 or D2 agonist was tested, the opposite saccule was used to test the other agonist. The sequence of agonists (SKF-38393 & quinpirole) was counterbalanced to control for order effects and accounted for in the statistical model. Because of the limited number of fish available due to difficulty of procurement, both saccules were also used in a subset of fish in the winter (N = 6) to compare the effect of dopamine and vehicle. As with agonist-treated summer fish, the order of treatment was counterbalanced. Potentials were recorded with glass microelectrodes (3-6 MΩ) filled with 3 M KCl that were positioned ~2.5 mm from the saccular epithelium within the medial/caudal region. A previous study found no regional differences in dopaminergic innervation across the saccule [22]. Potentials were amplified 100x (Getting 5A), band-pass filtered (130-3000 Hz, Stanford Research Systems SR 650) and passed to a digital signal processing lock-in amplifier (Stanford Research Systems SR830) and recorded to a computer. The lock-in amplifier DC output (RMS) is proportional to the component of the signal whose frequency is exactly locked to the reference frequency, which was set to the second harmonic of the stimulation frequency. This is because the maximum evoked potential from the teleost saccule is a doubling of the stimulus frequency due to the nonlinear response of hair cell populations with opposing polarities, which is characteristic of teleost fishes [14, 68, 87, 88]. Noise at frequencies outside of the reference are rejected by the lock-in amplifier and do not affect the potential recordings. Data acquisition and stimulus timing were controlled by custom MATLAB scripts. Single tone 500 ms stimuli were presented in repetitions of 8, at a rate of one every 1.5 s. Frequencies tested were 75, 105, 165, 205, 265, 305, 365 and 405 Hz across both seasons, and 505, 605, 705, 805, 905 and 1005 Hz for summer fish, and were presented in random order. Background noise measurements were averaged from 8 recordings in the absence an auditory stimulus. To characterize threshold tuning curves, stimuli were presented first at 130 dB re: 1 μPa, then in alternating ascending and descending increments of 3 dB, from 88 to 154 dB (the dynamic range of our playback system). Stimulus intensity was calibrated at the beginning of each experiment, using a hydrophone at the position of the fish's ear. Threshold was designated as the lowest stimulus level at each frequency that evoked a response greater than two standard deviations above the background noise measurement. Collection of tuning curves took 20-30 minutes. Measurements of evoked potentials and the resulting tuning curves represent the summed activity of a large population of hair cells and likely reflect the overall impact of dopamine modulation on transduction of auditory stimuli over a timescale of minutes to an hour. We cannot rule out transient effects of dopamine signaling on transduction at the level of individual hair cells or over shorter time courses.

qPCR

Immediately following completion of saccule potential recordings, fish were moved to an ice block, saccular epithelia were rapidly dissected out, trimmed of connecting nerve in ice cold buffer, transferred to RNAlater and incubated overnight at 4°C. Tissue was then stored at −80°C until use. RNA was isolated from individual saccular epithelia (2 per animal) using a Quick-RNA MicroPrep kit (Zymo Research). Tissue was pretreated with proteinase K (Zymo Research) and manually homogenized prior to RNA purification, followed by DNase treatment (Zymo Research). RNA quality and quantity were evaluated with a NanoDrop 2000 (Thermo Scientific). RNA from saccules for each individual was pooled and first-strand cDNA was synthesized from 1.05 μg RNA using SuperScript III RT (Invitrogen).

Relative quantitative real-time PCR (comparative Ct method) was used to compare dopamine receptor transcript expression between winter and summer using gene-specific primer pairs. Sequences for midshipman dopamine receptor subtypes were identified by querying two saccule-specific transcriptomes [33, 34]. Identification of specific receptor subtypes was achieved by aligning the sequences with published phylogenetic trees of D1-family and D2-family vertebrate protein sequences [32] (Figure S3) using Geneious (10.1.3). Although zebrafish have 14 dopamine receptor subtypes [32], querying transcriptomes from hindbrain [89] and preoptic area [90] midshipman tissue did not reveal additional dopamine receptor transcripts beyond the ones we identified in the

saccular transcriptomes. This suggests that midshipman may only possess genes for 7 dopamine receptor subtypes. Primers were designed using Geneious (10.1.3) and synthesized by Sigma-Aldrich (Table S1). All reactions, including no template controls, were run in triplicate on a StepOnePlus Real Time PCR systems (Applied Biosystems) using the sample maximization method [91]. Each well contained the following: 5μl 2x Power SYBR Green PCR Master Mix (Applied Biosystems), 1 μl forward and reverse primer, 2 μl H_2O, and 1μl cDNA. Relative transcript levels were normalized using 18 s rRNA, a transcript that has been shown not to vary between seasons in the midshipman saccule [26, 92].

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed in R (3.5.1) with assistance from the City University of New York Quantitative Research Consulting Center. A p value < 0.05 was considered significant. Unless indicated otherwise in figure caption, all error bars depict means with 95% confidence intervals. Plots were generated with either R or Graphpad Prism (7.0a). Threshold data were fit with linear mixed models implemented with the R package lme4 [93]. Separate models were constructed to evaluate the effects of dopamine dose in the summer (Figure 2A), drugs (Figures 2D–2F) on thresholds, with frequency, treatment condition and their interaction entered as fixed effects and subject as a random effect. A mixed model was used to evaluate seasonal differences (Figure 3A) with frequency, treatment condition, season, and their interaction entered as fixed effects and subject as a random effect. For models that included subjects where both saccules were utilized (effect of agonists and effect of dopamine in winter), side was included as a random effect nested within subject. Furthermore, any possible order effects were accounted for by including order of treatment as fixed effect. The absence of thresholds greater than 405 Hz for many summer females in the 5 mM dopamine, 50 mM dopamine and quinpirole groups was likely due to thresholds being raised above the maximum sound level of our speaker by the treatment. One advantage of linear mixed models over traditional repeated-measures ANOVA is their ability to handle missing data points from individual subjects without the need to discard all of a subject's data, however, missing values must be ''missing-at-random'' and not due to systemic influence [94]. Missing data at higher frequencies cannot be considered as ''missing-at-random'' and so we limited our models from 75 to 405 Hz. To evaluate the effects of dopamine and receptor agonists on frequencies above 405 Hz, we used survival models to compare the reduction of responses as a function of both frequency and treatment (Figure S2). We fit Cox mixed-effects models in R with the Coxme package. We fit separate survival models for dopamine dose and drugs, with highest frequency with an obtained threshold for each individual as the outcome variable, treatment condition as a fixed effect and subject as a random effect. Post hoc pairwise comparisons for the effect of dopamine on thresholds in the winter were adjusted with the Bonferroni correction. ANOVA was used to compare average threshold changes indcued by dopamine dose and quinpirole (Figure 2B) and ANCOVA was used to compare average threshold changes induced by dopamine in low versus high frequency ranges between summer and winter fish (Figure 3B). Mann-Whitney U tests with Bonferroni corrections were used to compare dopamine receptor subtype transcript expression between summer and winter. To evaluate the relationship between D2a expression and thresholds, we utilized linear mixed models with frequency and D2a expression as fixed effects and subject as a random effect. Individual models were constructed within and across seasons. Statistical significance values for all mixed models were determined using the lmerTest package in R [95], fitted used restricted maximum likelihood (REML) and Satterthwaite approximation [96].

DATA AND SOFTWARE AVAILABILITY

Custom MATLAB scripts used in this report are available at the following URL: http://forlanolab.com/?page_id=871.