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Delta opioid receptors affect acoustic features of song during vocal learning in zebra finches

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Abstract

Delta-opioid receptors (δ-ORs) are known to be involved in associative learning and modulating motivational states. We wanted to study if they were also involved in naturally-occurring reinforcement learning behaviors such as vocal learning, using the zebra finch model system. Zebra finches learn to vocalize early in development and song learning in males is affected by factors such as the social environment and internal reward, both of which are modulated by endogenous opioids. Pairs of juvenile male siblings (35-day-old) were systemically administered a δ-OR-selective antagonist naltrindole or vehicle (controls) for a period of 10 days. The acoustic structure of songs differed across treated and control groups at adulthood (120 days). Naltrindole-treated birds had a significantly lower pitch, mean frequency, and frequency modulation than controls, whereas there was no difference in the number of songs in naltrindole-treated and control siblings. Since the opioid and dopaminergic systems interact, we decided to study whether blocking δ-ORs during the sensitive period led to changes in dopaminoceptive neurons in Area X, a song control nucleus in the basal ganglia. Interestingly, compared with controls, naltrindole-treated birds had higher numbers of DARPP-32-positive medium spiny neurons and potentially excitatory synapses in Area X. We show that manipulating δ-OR signaling during the learning phase resulted in alterations in the acoustic features of song and had long term effects on dopaminergic targets within the basal ganglia in adulthood. Our results suggest that endogenous opioids regulate the development of cognitive processes and the underlying neural circuitry during the sensitive period for learning.

Keywords Zebra finch, Vocal learning, Sensitive period, Delta-opioid receptors, Naltrindole, DARPP-32

Background

Birdsong is a socially transmitted behavior [1, 2]. Social factors such as the presence of siblings [3], adult tutors [4], and maternal responses [5] during the learning phase play a major role in vocal acquisition. Whereas the role of an enriched social environment is well studied in songbirds [6–8], there is limited information about neuromodulators that regulate this socially guided form of learning. One such class of neuromodulators are the opioid peptides including endorphins, enkephalins, and dynorphins [9]. Endorphins preferentially bind to mu-opioid receptors (μ-ORs), enkephalins bind to the delta-opioid receptors (δ-ORs) and dynorphins bind to the (κ-ORs). Additionally, each endogenous opioid peptide can also act by binding to other types of opioid receptors [10–12]. Furthermore, μ- and δ-ORs assign positive affective states to food and addictive behaviors [13] and κ-ORs are known to be anxiogenic and anti-addictive [14]. Whereas earlier studies have studied the role of μ-ORs on vocalization and the motivation to sing

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[15–17], the role of ORs in vocal learning has not been explored.

We decided to focus on delta opioid receptors (δ-ORs) which are expressed in brain areas regulating associative learning, decision-making, and emotions [18–20]. It is thought that δ-ORs decrease anxiety and help in learning the association between a drug and its addictive effects [21–23]. Furthermore, an intricate coupling of opioid modulation and dopamine [24] suggests that δ-ORs may affect cognitive functions. For example, in chickens, δ-ORs were implicated in learning using passive avoidance tasks [25, 26]. In rodents, in-vivo microdialysis of the δ-OR agonists [D-Pen2,5]-enkephalin (DPDPE) and [D-Ser2]Leu-enkephalin-Thr6 (DSLET) in the nucleus accumbens led to an increase in the release of dopamine, which was abolished by specific δ-OR antagonists [27]. Studies have also demonstrated that δ-ORs and dopaminergic receptors (D1R) were co-expressed in the dendrites of medium spiny neurons (MSNs) of the dorsolateral striatum [27–30] and in the shell of nucleus accumbens in rodents [31]. These findings underlie the fact that the opioid system plays a role in enhancing dopaminergic neurotransmission and the rewarding properties of drugs such as cocaine [32, 33], although as a caveat, knockdowns of both μ- and δ-ORs did not have significant effects on the motivation to obtain rewards [34]. Besides interactions between δ-ORs and dopaminergic circuits, Bertran-Gonzalez et al. (2013) reported that high levels of δ-ORs were expressed by cholinergic interneurons in the nucleus accumbens shell in mice trained to associate a food reward with an external stimulus and the level of association determined the degree of expression of δ-ORs and modulated the choice for a rewarding stimulus [35]. Mutant mice lacking δ-ORs have impaired hippocampal learning, wherein they perform poorly on place recognition tasks. In contrast, striatal-based learning is strengthened in δ-OR knockout mice suggesting that this opioid receptor subtype plays a role in balancing striatal and hippocampal function [36]. Taken together, these findings suggest that δ-ORs and dopaminergic system interact with each other and modulate various cognitive functions.

It is therefore possible that vocal learning is modulated by the opioid and dopaminergic systems. Vocal learning in songbirds and humans is regulated by a fine balance between striatal and cortical function [37, 38]. In songbirds such as zebra finches, males learn to sing from their fathers during a sensitive phase in development. Juvenile birds acquire their vocalizations during two overlapping phases: sensory and sensorimotor. The sensory phase lasts until ~60 days post hatch (dph) during which juvenile birds form a mental template or memory of their father's song [39]; the sensorimotor phase begins at ~25-30dph, when birds start producing a soft, structurally variable subsong [40]. Young birds use auditory feedback to correct this song and produce 'plastic' songs which, although malleable, possess an adult-like acoustic structure. These songs become fixed or stereotyped in adulthood (~120dph; reviewed in [41]).

Song behavior is controlled by specialized brain areas connected through two overlapping neural pathways: the anterior forebrain pathway (AFP) and the vocal motor pathway (VMP). The AFP is essential for vocal learning [42] and resembles mammalian basal ganglia thalamocortical loops [43]. This pathway connects the pallial song control nucleus HVC to a nucleus in the basal ganglia (Area X). Area X projects to the dorsolateral nucleus of the medial thalamus (DLM) which loops back to the lateral magnocellular nucleus of the anterior nidopallium (LMAN) [44–46]. Additionally, LMAN forms a loop within the AFP by projecting to Area X [47, 48]. The AFP is also connected to the VMP via projections from LMAN to a motor cortical nucleus, the robust nucleus of the arcopallium (RA) [42, 49]. The VMP, which is required for vocal production, consists of a group of neurons in HVC which project to RA, distinct from neurons in HVC which project to Area X [50, 51]. Anatomical studies have shown that these song control areas express high levels of opioid receptors during the vocal learning phase as well as adulthood [52, 53].

Opioids are capable of modulating both the number and quality of songs [15, 16]. Since singing also induced gene expression of the preferential δ-OR ligand enkephalin in Area X and HVC of both juvenile and adult zebra finches [54], we became interested in exploring the involvement of δ-ORs in vocal learning in young zebra finches. This form of reinforcement learning is regulated by an interplay of social factors and internal reward, suggesting that it may be regulated by neuromodulators, such as the endogenous δ-OR system (reviewed in [55]). For these experiments, we pharmacologically manipulated δ-OR signaling at the beginning of the sensorimotor phase of learning in juvenile zebra finches by systemically administering naltrindole, an opioid antagonist that is highly specific for δ-ORs [56]. Zebra finches are an excellent model system for this study since juvenile birds learn their songs from their fathers and therefore, the tutors and their offspring have similar song elements, providing valid controls for this study [57]. We sought to establish whether blocking δ-OR signaling for a very short duration during the sensitive period for learning induced changes in the song control circuitry or in the spectro-temporal features of song when birds attained adulthood. Briefly, our results demonstrated that blocking δ-ORs during the sensitive period led to changes in the spectral features (pitch, mean frequency and frequency modulation). We also found that this manipulation led to an increase in the medium spiny neurons, which are

dopaminoceptive and excitatory synapses in Area X, which are known to originate in glutamatergic neurons in HVC and LMAN [58, 59].

Methods

Animals

All experiments were approved by the Institutional Animal Ethics Committee at the National Brain Research Centre (NBRC), Manesar, in accordance with the guidelines laid down by the Committee for the Control and Supervision of Experiments on Animals (CCSEA), India (protocol number: NBRC/IAEC/2013/85). All birds used in this study were obtained from a breeding colony maintained by the Animal House at NBRC, Manesar. Nine pairs of juvenile male zebra finches (35dph) were used for this study, each pair consisting of male siblings/clutch-mates. Birds were housed within the aviaries with their parents during the course of the experiment to ensure normal social interactions. The aviaries housed twelve pairs of adult birds (> 120dph) with three to four offspring per pair. At 50dph, experimental birds were removed from the aviary and housed in separate cages in auditory and visual contact with their parents.

Treatment paradigm

The selective non-peptidic δ-OR antagonist naltrindole (cat: 7040, CAS number: 111469-81-9; Tocris Bioscience, Ellisville, MO, USA) was dissolved in 0.9% saline to obtain a stock solution of 2.2mM (1 mg/ml). The drug dose was calculated according to the weight of the birds and the stock was diluted to a volume of 100 µl for injections. In each experimental set, one bird was administered naltrindole intramuscularly (10 mg/kg body weight) once every day for 10 days, starting at 35dph to 45dph, whereas its control sibling was administered 0.9% saline (vehicle) for the same duration. We decided to use the 10 mg/kg body weight dose based on a dose-response curve for different doses (1 mg, 2.5 mg and 5 mg/kg body weight versus 0.9% saline in controls) of naltrindole in adult male zebra finches ($n = 28$; details provided in Suppl. Information). Since there was a significant decrease in FD songs of naltrindole-treated males after administration of the 5 mg/kg weight dose (Fig. S1a), we decided to use 10 mg/kg body weight dose (greater than the 5 mg/kg body weight dose) for a short duration (10 days) in juvenile males. Birds were observed for 30 min after the injections for any signs of stress or trauma. For the first 5–10 min of this duration, both controls and treated birds remained in their nest boxes or perched atop them. None of the experimental birds showed any signs of sickness or lethargy and interacted normally with other birds during the course of treatment/vehicle administration.

Behavioral recordings

Starting from 80dph to 120dph, female-directed songs (FD songs) were recorded from both control and treated birds during the first half of the day at ten-day intervals. Experimental birds were removed from their cages and placed individually in a cage (dimensions: 12 × 15 × 11 inches; length × breadth × height), which was placed in a sound-attenuated chamber. A cage containing 3–4 female birds were housed in a separate cage placed in visual and auditory proximity to that of the male birds. We decided to only record FD songs of control and treated birds, since these are easily elicited during uniform time intervals by placing females in a cage adjacent to the male birds. Songs directed towards the females were recorded using a microphone (Sennheiser e614, M-track quad audio device). Software, namely Goldwave (Version 5.10 Goldwave Inc.) and Audacity (Version 3.14-beta) were used to record songs and calls. To determine whether male birds were singing female-directed songs, video recordings were simultaneously performed using a Handycam (Sony; DCR-SR67E E37; [16]). To differentiate between FD and UD song, we examine the video recordings of all experimental birds and counted only those wherein birds were facing the females and singing while they were adjacent to the female cage. Furthermore, we also observed the dance-like movements including tail flicks and head turns which accompanied FD song [60]. Throughout the duration of the experiment and until 120dph, the experimental birds were situated in an enriched social environment in auditory and visual proximity with approximately 50 other birds in the aviary.

Song analysis

Songs recorded between 80 and 120dph were segmented using Sound Analysis Pro (SAP) [(SA+), Ver.-1.02] [61] by setting the Wiener entropy at 3.6 for all birds. This was followed by setting the amplitude for each bird, such that the software recognized individual notes in the songs while ensuring that background sounds were not included. We did not smooth any feature of the songs during our analysis. The 'minimum stop duration' were set to 7ms and 'bout ends' were set to 100ms.

Song quality for all 9 sets of siblings was assessed for temporal features such as syllable duration and inter-syllable interval (ISI), and for frequency-based/spectral features including mean pitch [henceforth termed pitch], mean frequency (MF) and mean pitch goodness, amplitude and amplitude modulation (AM), FM, and wiener entropy. Spectro-temporal features and similarity scores were also compared across tutors and tutees (aged 120 dph) to study how much of the fathers' songs had been copied by each sibling (SAP software). For this analysis, songs of control and naltrindole-treated siblings which had the same number of syllables were selected to

analyze similarity between the individual syllables. We ensured that each syllable in these motifs were distinct and there were no overlapping background sounds (for example, female calls). The SAP software uses percent similarity (to measure the percentage of similar syllables between the offspring and tutor's songs), mean accuracy (to measure similarity between individual frequency traces) and sequential match (temporal order of syllables) to calculate similarity scores. Measurements of the spectral features including pitch, FM, AM, goodness of pitch and Wiener entropy were transformed for 10ms intervals are transformed into median absolute deviations from the mean (MAD), which are units of statistical distances. These values are used to calculate the Kolmogorov-Smirnov (KS) distance (the Euclidean distance between the cumulative histograms of spectral and temporal features of the father's and offspring's songs). The larger the KS distance, the farther apart are the feature values between the compared motifs.

We analyzed 15–20 songs/bird for each developmental time point. Songs were randomly chosen and only excluded if cage noise or female calls overlapped the song. The temporal order of notes was manually scored. Stereotypy was analyzed by averaging sequence linearity (the order of syllables within a motif) and consistency (the predominant transitions for a particular syllable) [62]. The formulae for calculating these measures are as follows:

$$Linearity = \frac{number\ of\ different\ syllables - 1}{number\ of\ syllable - to - syllable\ transitions}$$

Consistency = ∑ (dominant transition/all transitions)/N; wherein N = number of syllables.

Stereotypy = Linearity/Consistency for each bird.

All variations in song were included, whereas introductory notes were excluded for this analysis. We also counted the number of introductory notes, motifs, and motif variants.

Histology

Tissue sectioning

Transcardial perfusion was performed after birds were deeply anesthetized with an overdose of intramuscular injections of ketamine (100 mg/kg body weight) and xylazine (1 mg / kg body weight), until they were areflexive to hard toe pinches. After initial perfusion with saline (0.74%), all birds used in these experiments were perfused with 4% paraformaldehyde (PFA) after the last song recordings were obtained (at 120dph). Serial coronal sections (5 series; 40 μm thick) were cut using a cryostat (Leica, CM 3050 S). The first series of sections from each brain was stained with Nissl (Thionin) to identify various brain regions. Other series were used for immunohistochemistry (IHC) for DARPP-32 and to detect synapses [(using double-labeling for Synaptotagmin and Post-Synaptic Density-95 (PSD-95), see below]. Whole brain lysates from adult male zebra finches sacrificed by an overdose of halothane were used to test the specificity of the antibodies used for IHC for zebra finch brain tissue.

Immunohistochemistry

DARPP-32

To visualize DARPP-32, we used sections mounted on gelatin coated slides [63]. Antigen retrieval was performed by incubating sections in an antigen unmasking solution at 80 °C (citrate buffer, pH 6.0, H-3300, Vector Laboratories, Burlingame, USA). After cooling to room temperature, sections were rinsed with 0.01 M PBS and incubated in 2% H_2O_2 in PBS for quenching. After rinsing in PBS, sections were incubated in 0.1% Triton X to increase permeability, followed by incubation for 3 h in a solution containing 1% BSA (bovine serum albumen) and 5% normal goat serum (NGS, Vector Laboratories, Burlingame CA; S-1000), made in 0.01 M PBS. This was followed by incubating sections in a solution containing the primary antibody [anti-DARPP-32 antibody (1:500; ab-40801, abcam, USA, raised in rabbit; RRID: AB_731843)], 3% NGS, 1% BSA, made in 0.1% Triton-X in PBS for 18 h. Sections were then rinsed with PBS and incubated in a solution containing the secondary antibody (biotinylated anti-rabbit IgG produced in goat; Vector Laboratories, Burlingame, USA, BA-1000; 1:250 and 1% NGS) for 2 h. This was followed by rinsing in PBS and incubation in a solution containing the avidin-biotin complex (1:50, ABC reagent; Vectastain Elite ABC HRP kit, Vector Laboratories, Burlingame, USA; PK-6100) at room temperature for 2 h. After final rinses with PBS, the Nova Red peroxidase kit (Vector Laboratories, Burlingame, CA, SK-4800) was used to develop sections according to the manufacturer's instructions.

Synaptotagmin and Post-Synaptic Density-95 (PSD-95)

Sections mounted on gelatin-coated slides were rinsed with PBS and quenched as described above. Sections were then incubated in a blocking solution containing 5% Normal Goat Serum (NGS; S-1000, Vector Laboratories, Burlingame, CA) and 1% bovine serum albumin (BSA; A-7906, Sigma-Aldrich) for 3 h, which was followed by incubation in a solution containing a combination of anti-PSD-95 (1:200; ab9708, Abcam; polyclonal; a marker for post-synaptic scaffolding proteins in excitatory neurons and anti-Synaptotagmin antibody (1:200; MAB5200 Millipore, monoclonal; a calcium-sensitive pre-synaptic marker 3% NGS, 0.3% Triton X-100 and 1% BSA) for 18 h. The slides were rinsed with PBS, after which they were incubated in a secondary antibody solution

containing Goat Anti-Rabbit IgG Alexa Fluor 488 (1:300; A-11008, Invitrogen) and Goat anti-Mouse IgG, Alexa Fluor 594 (1:300; A-11005, Invitrogen) for 2 h. Sections were rinsed in PBS and mounted with VECTASHIELD Anti-fade mounting medium containing DAPI (H-1200, Vector Labs). Fluorescence images of the sections were captured using a confocal microscope (LSM 510 Meta, Carl Zeiss, Germany; see below).

Western blots

Total proteins from brain tissue obtained from adult male zebra finches were blotted on to a nitrocellulose membrane. Blocking was performed by incubating the membrane with BSA in TBST (5%), after which the membrane was incubated with the anti-DARPP-32 antibody (1:5000; ab-40801, Abcam; monoclonal; made in rabbit; RRID: AB_731843) for 16 h at 4 °C. For detecting PSD-95 and Synaptotagmin proteins, membranes were incubated with anti-PSD-95, (ab9708, Abcam, USA; 1:2000 dilution; made in rabbit) and anti-synaptotagmin (MAB5200, MERCK Millipore; USA; 1:1000 dilution; made in mouse), respectively, also for 16 h at 4 °C. This was followed by incubating the membrane in a solution containing the alkaline phosphatase-labelled anti-rabbit antibody (1:1000; raised in goat, PI-1000; Vector Laboratories, Burlingame, CA; for DARPP-32 and PSD-95) and the alkaline phosphatase-labelled anti-mouse antibody (1:1000; raised in horse, Vector Laboratories, Burlingame, CA) for 1 h. The ECL chromogenic method was then used to detect bands specific for DARPP-32.

Stereology

Major song control nuclei (LMAN, Area X, HVC and RA) in control and naltrindole-treated birds ($n = 9$ sets) were outlined in their entirety, using the Stereoinvestigator software (MBF Bioscience). The Cavalieri estimator probe was used to estimate the volume of each nucleus in mm^3. Of the 5 series of cryosections (40 µm) obtained from these birds, volume estimation was performed on the first section, which was stained with Nissl. A grid spacing of 10 µm used to place grids generated by the software over the song control nuclei, which were observed at a magnification of 40X. Points in the grid overlying the song control nuclei in each bird were then marked. The volume of each song control nucleus was given by the formula:

$$\begin{aligned} &Volume\ of\ a\ structure \\ &\quad = Total\ number\ of\ points\ marked \\ &\quad \times Distance\ between\ points\ in\ XY \\ &\quad \times Distance\ between\ points\ in\ Z \\ &\quad (wherein\ X,\ Y\ and\ Z\ represent\ the\ three\ axes) \end{aligned}$$

For the remaining analyses (counts of DARPP-32-labeled neurons in Area X and LMAN and synapses in Area X), 5 sets of birds were randomly selected from amongst the original 9 sets used for this analysis.

One of the series of cryosections at the level of the anterior forebrain was used to count the number of DARPP-32-positive cells throughout the extent of LMAN and Area X from 5 sets of randomly selected experimental sibling pairs. We decided to use the optical fractionator method, which estimates the total number of cells in any brain region, by randomly sampling cells in three dimensions using counting frames placed at regular intervals covering LMAN and Area X in all sections in which they appeared (Stereoinvestigator; MBF). The optical fractionator probe was used by viewing sections under the 100x objective and using a counting frame size of 150 µm x150µm. The thickness of the section was measured at each counting site of the optical fractionator probe. The formula for estimating the number of neurons using the optical fractionator method is as follows:

$$N = \sum Q^{-} \times t/h \times 1/asf \times 1/ssf$$

Q^{-}: Particles counted.
t: Section mounted thickness.
h: Counting frame height.
asf: Area sampling fraction (counting frame/grid size).
ssf: Section sampling fraction.

Puncta analysis

The zebra finch AFP consists of topographically organized 'core' areas (LMANcore, Area X, dorsolateral DLM and RA) and 'shell' areas (LMANshell, MSt, ventromedial DLM and AId [44]. Since Area X was the only 'core' area with a substantial population of DARPP-32-labelled neurons and there was an increase in their number following naltrindole administration (see Results), we decided to quantify the changes in synapses in this region. For this analysis, the number of synapses (points of co-localization) was counted in naltrindole-treated birds versus their vehicle-treated siblings ($n = 5$ sets of siblings used for DARPP-32 counts. A confocal microscope (LSM 510 Meta, Carl Zeiss, Germany) equipped with an Argon laser and a HeNe laser and was used to image synaptotagmin and PSD-95-positive puncta using a Plan-Apochromat 63x/1.4 NA oil immersion lens. Alexa Fluor 488 was visualized using a 505–550 band pass filter whereas Alexa Fluor 594 was captured in the 560 band pass range. Z-stacks (0.33 µm thick optical sections) at 512 × 512 pixels were captured for further analysis, each of which consisted of 15 sections. Three sections were merged into one maximum intensity projection (MIP). In this way, 5 MIPs were generated from each stack with each

MIP being ~ 1 µm in thickness. The Image J plugin, Iterative Deconvolve 3D, was used to deconvolve the images, which was followed by quantifying puncta using the Puncta Analyzer plugin [64].

Statistical analysis

All statistical tests were performed using software from SigmaPlot (versions 12 and 15; Systat Software Inc., USA). Two-Way Repeated Measures analysis of variance (Two-Way RM ANOVA) was used to test the interaction between developmental age, treatment, and values of number of songs, spectral and temporal features of control birds and their naltrindole-treated siblings. Of these, we found significant differences only in the spectral features (Fig. 1). This was followed by post-hoc analysis using the Bonferroni method. We also used the Benjamini-Hochberg (B-H) correction with an FDR (false discovery rate) of 0.05 for this data. While using this correction, p-values for sets of comparisons at each age were compared with their critical B-H value, obtained by the formula (i/m)Q, where 'i' = rank of individual p-value, m = total number of correlations and Q (FDR) = 0.05 [65].

Furthermore, changes in the number of FM and H-stack syllables in the songs of control and treated birds (Figs. 2 and 3), changes in linearity, consistency and stereotypy (Fig. 4), changes in the number of DARPP-32-positive neurons (Fig. 6) and differences in the number of synapses in Area X between control and treated birds (Fig. 7) were analyzed using the Student t-test and the Mann-Whitney Rank Sum test (MWU) was used wherever data were not normally distributed. Bar graphs represent mean values and error bars represent standard deviation. Each point in the scatter plots represents the average value of data for one bird. One-way ANOVA

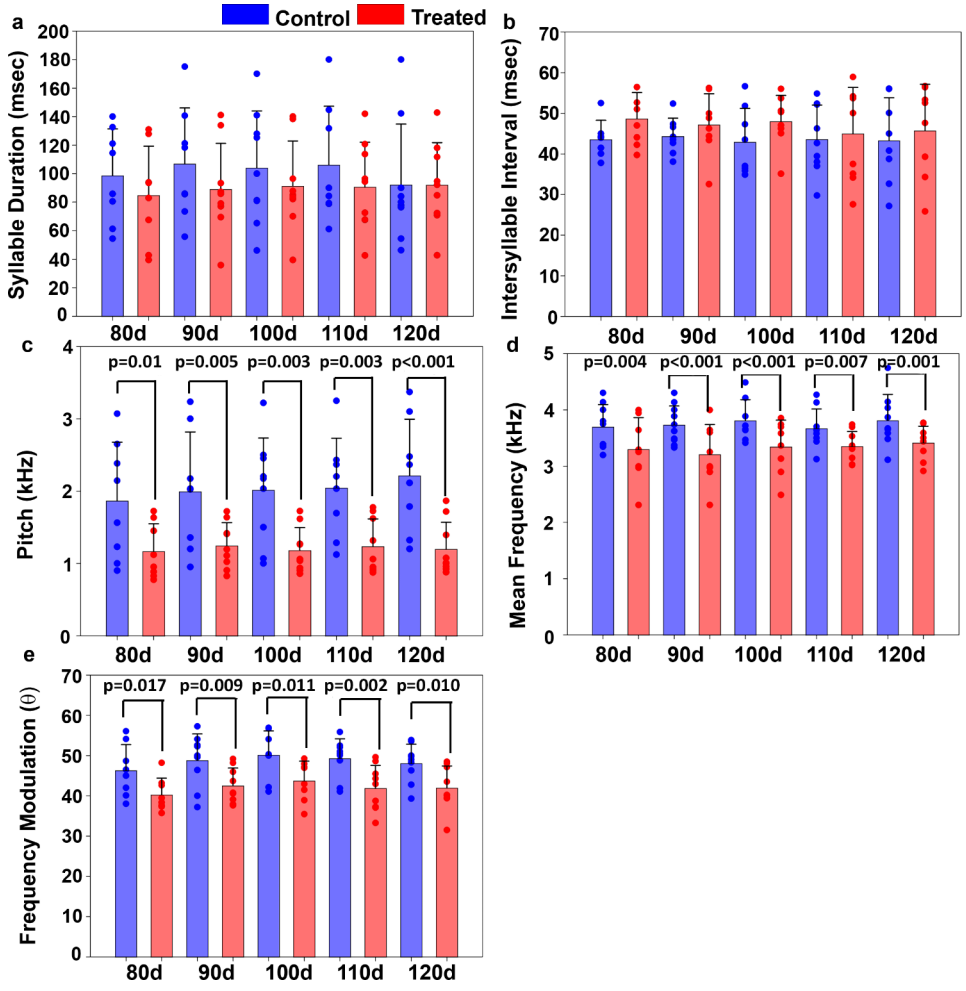

Fig. 1 Quantitative analysis of spectral and temporal features of motifs sung by naltrindole-treated and control birds from 80dph to 120dph. The bars represent means, the error bars represent standard deviations and dots represent mean feature value per bird. (**a**) Birds treated with naltrindole during the sensitive period of learning tended to have shorter syllables than their control siblings, although these differences were not statistically significant. There were no significant differences between (**b**) intersyllable intervals of control and treated birds. There was a significant effect of treatment with δ-OR antagonist naltrindole on (**c**) pitch, (**d**) mean frequency and (**e**) frequency modulation, all of which were significantly lower compared with the same measures from vehicle-treated siblings. Results of Bonferroni post-hoc tests for these measures are shown above the bars representing different ages

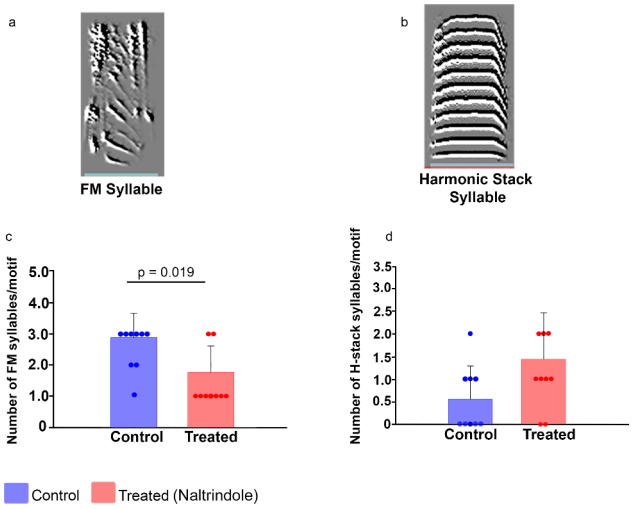

Fig. 2 (**a**) Sonogram representing an exemplar of an FM syllable. These are complex syllables with intricate frequency traces. (**b**) Sonogram representing a harmonic stack syllable which is simpler than FM syllables and consists of frequency traces arranged in a stack above the fundamental frequency. Both these types of syllables were used to measure the motif complexity of the naltrindole-treated birds and their control siblings. Figures show average number of syllable types produced by the (**c**) control and (**d**) treated birds. Each point in the scatter plot represents data for one bird for the number of FM or H-stack type syllables per motif. (**c**) There was a significantly greater number of FM syllables in the songs of controls, whereas (**d**) songs of birds administered the δ-OR antagonist naltrindole tended to have a greater number of harmonic stacks

was used for comparing spectral and temporal features between siblings and tutors. The Holm-Sidak method was used for pairwise multiple comparisons to test for statistical significance (Fig. 5).

Results

Administration of naltrindole during the sensitive period of learning affects the acoustic quality and stereotypy of adult song

We analyzed the spectral and temporal features of songs sung by control and naltrindole-treated zebra finches between 80d and 120d post-hatch to study whether blocking δ-ORs during the sensitive period of song learning led to changes in the stereotyped song during adulthood [60]. There were no significant differences as a factor of age using Two Way Repeated Measures ANOVA for any of the spectral and temporal features of song sung by control and naltrindole-treated birds during this period. Although there was an overall effect of naltrindole treatment on syllable duration [$n = 11$; $F = 4.499$, $p = 0.041$; degrees of freedom (df) = 1], there were no significant differences between control and treated birds at any age for this measure. Furthermore, there were no significant differences with either age or naltrindole treatment as factors for ISI (Fig. 1a, b).

Amongst the spectral features, the pitch of the songs of control birds was significantly higher than in naltrindole-treated birds with treatment as a factor ($n = 11$; $F = 51.54$, $p < 0.001$; $df = 1$) at each time-point used in our experiments ($t = 7.18$; $p < 0.001$ for Treatment; Bonferroni post-hoc tests; 80 days: $t = 2.739$; $p = 0.01$; 90 days: $t = 3.024$; $p = 0.005$; 100 days: $t = 3.203$; $p = 0.003$; 110 days: $t = 3.157$; $p = 0.003$; 120 days: $t = 3.935$; $p < 0.001$; Fig. 1c; also see Suppl. Table S1). We also found that the MF of treated birds' songs was significantly lower than that of the songs of control birds with treatment as a factor ($n = 11$; $F = 64.81$, $p < 0.001$; $df = 1$). Pairwise multiple comparisons also demonstrated these differences ($t = 8.051$; $p < 0.001$ for Treatment; Bonferroni post-hoc tests; 80 days: $t = 3.064$; $p = 0.004$; 90 days: $t = 4.562$; $p < 0.001$; 100 days: $t = 3.942$; $p < 0.001$; 110 days: $t = 2.852$; $p = 0.007$; 120 days: $t = 3.544$; $p = 0.001$; Fig. 1d; Suppl. Table S1). Lastly, the levels of frequency modulation (FM) were significantly higher in control birds' songs than in treated birds' songs ($n = 11$; $F = 38.27$, $p < 0.001$; $df = 1$) with treatment as a factor. This data was supported by pairwise comparisons at different ages (Treatment: $t = 6.186$; $p < 0.001$; Bonferroni post-hoc tests: 80d: $t = 2.491$; $p = 0.017$; 90d: $t = 2.764$; $p = 0.009$; 100d: $t = 2.658$; $p = 0.011$; 110d: $t = 3.256$; $p = 0.002$; $t = 2.693$; $p = 0.010$; Fig. 1e; Suppl. Table S1).

To compare the types of syllables incorporated in the control and treated birds' songs, we counted complex FM syllables and the simpler harmonic stack (H-stack) type of syllables in songs of both sets of birds (Fig. 2a and b). We found that naltrindole-treated birds had significantly lower numbers of FM syllables (Fig. 2c; MWU = 14.5, $P = 0.019$) and incorporated a greater number of the H-stack type of syllables in their songs (Fig. 2d). For H-Stack syllables, values of pitch were slightly lower in songs of control birds (MWU = 15, $p = 0.027$, Fig. 3a), whereas for FM syllables, controls had a significantly higher pitch versus that of their control siblings (MWU = 27499, $p < 0.001$; Fig. 3a). The mean frequency was higher in controls for both H-stack as well as FM syllables (H-stack: $t = 2.45$, $p = 0.034$; FM: $t = 2.51$, $p = 0.023$; Fig. 3b), compared to that of their naltrindole-treated siblings. Whereas frequency modulation was not significantly different for the H-stack syllables in the two groups of birds, it was significantly lower in the songs of naltrindole-treated versus control birds for FM syllables ($t = 2.14$, $p = 0.047$; Fig. 3c). There were no differences in Wiener entropy of the H-stack and FM syllables sung by control and treated birds (Fig. 3d). Whereas there were no differences in the duration of H-stack syllables in control birds and their naltrindole-treated siblings, FM syllables were significantly longer in the songs of controls versus treated birds ($t = 2.571$, $p = 0.021$).

An analysis of song stereotypy demonstrated that linearity (MWU = 18, $p = 0.014$; Fig. 4a) and stereotypy (MWU = 17, $p = 0.04$; Fig. 4c) were significantly lower,

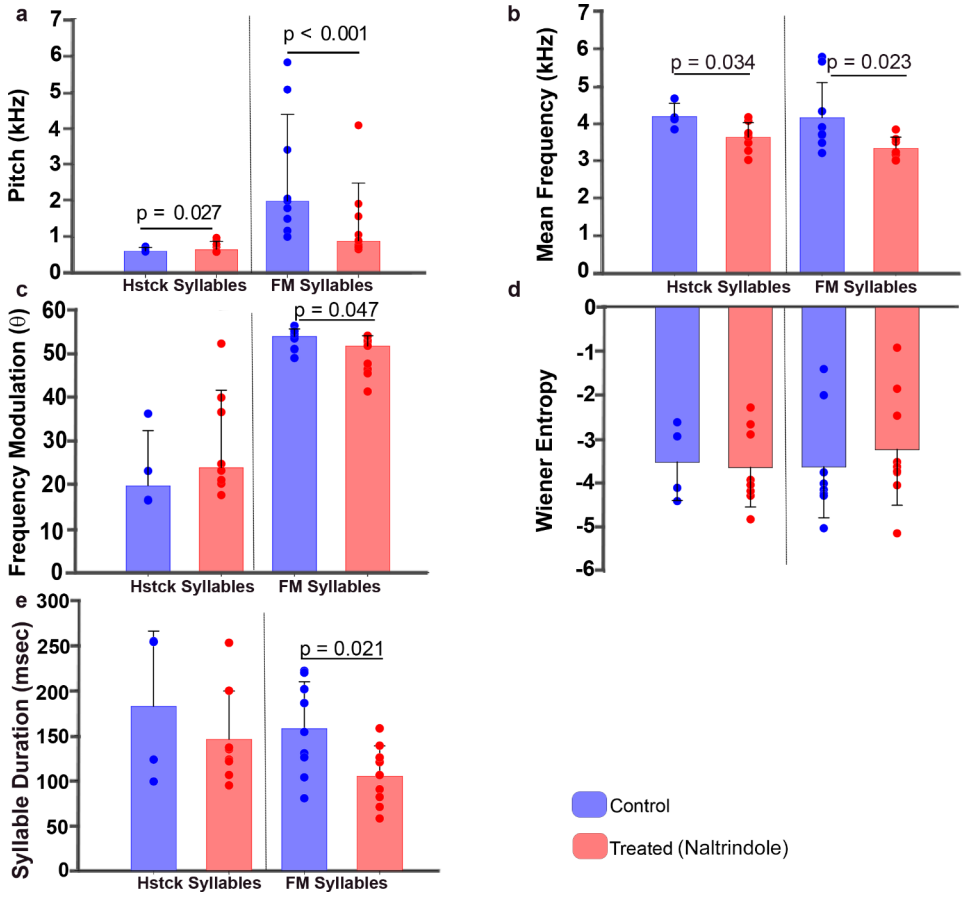

Fig. 3 Analysis of spectral and temporal features of harmonic-stack (Hstck) and FM syllables. Bars represent mean values; error bars represent standard error and dots represent feature value per syllable. (**a**) Frequency modulated syllables were produced at a higher pitch by controls than naltrindole-treated birds. For H-Stack syllables, values of pitch were slightly lower in songs of control birds. (**b**) The value for mean frequency obtained after analyzing both types of syllables was higher in songs of control birds, compared to those sung by their naltrindole-treated siblings (**c**) Frequency modulation was not significantly different for the H-stack syllables in the two groups of birds, but was lower in naltrindole-treated versus control birds for FM syllables. (**d**) No significant differences were observed between Wiener entropy values for control and treated birds for both types of syllables. (**e**) The frequency-modulated syllables produced by controls were significantly longer than those produced by treated birds. No such temporal difference was observed for the harmonic stack syllables sung by control and naltrindole-treated siblings

whereas there was no difference in the consistency (Fig. 4b) in treated birds' songs compared to those of controls. We also found that the average number of motifs per bout (Fig S2a), number of introductory notes per motif (Fig S2b), and total number of motif variants (Fig S2c) were not significantly different between control and treated birds.

Comparisons between the acoustic quality and syllable order of the songs of control and naltrindole-treated siblings and their fathers

The vocalizations of each set of control and treated siblings were compared with the common learnt template, that is, their fathers' songs (Fig. 5a). Comparisons of spectral features of pairs of siblings with those of their fathers revealed significant within-group variations for pitch [F (2, 23) = 4.725, $p = 0.019$]. The Holm-Sidak test for multiple comparisons showed that the pitch of the treated bird's song was significantly lower than that of controls ($p = 0.024$), and was lower but not significantly different from the father's songs [($p = 0.061$), Fig. 5b]. The MF was also significantly different between tutors and tutees [F (2, 23) = 4.30, $p = 0.026$], wherein MF of treated birds' songs was significantly lower than that of controls ($p = 0.045$) and their fathers ($p = 0.047$), but was not significantly different between that of the fathers and control birds [($p = 0.813$), Fig. 5c]. Lastly, there were significant within-group differences in FM [F (2, 23) = 5.355, $p = 0.012$]. Multiple comparisons for FM demonstrated that treated birds' songs had a significantly lower FM than those of controls ($p = 0.017$) and of the fathers ($p = 0.040$); but the difference was not significant between fathers and controls [($p = 0.644$), Fig. 5d]. Furthermore, there were no significant differences in the similarity (Fig. 5e) and Kolmogorov-Smirnov (KS) distance (Fig. 5f)

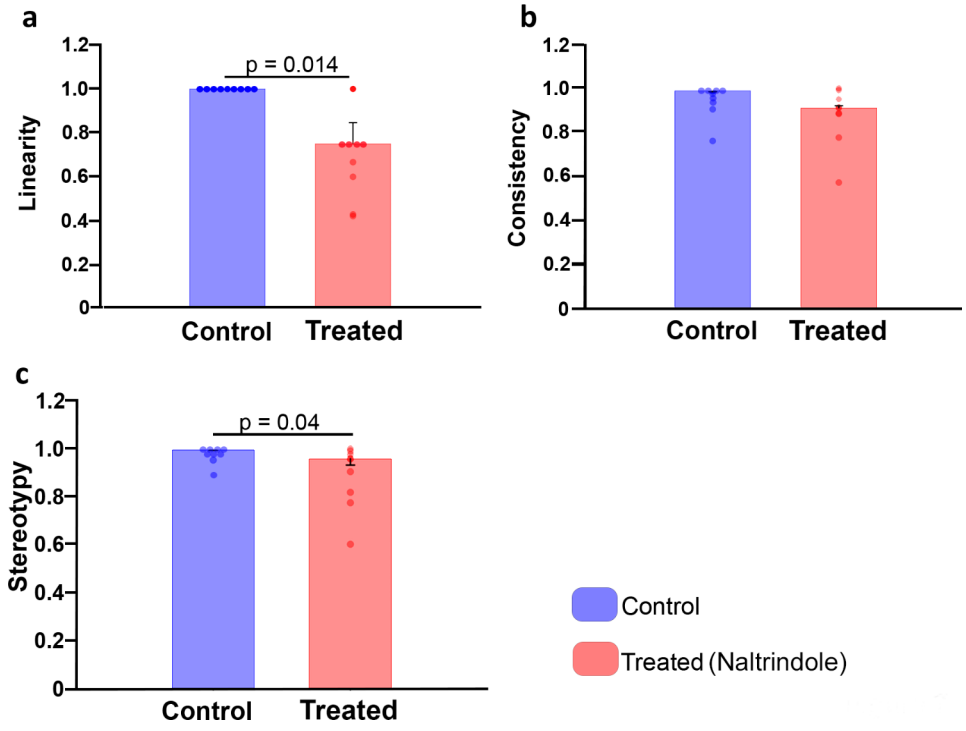

Fig. 4 Comparison of syllable order between control and naltrindole-treated birds. Bars graphs represent mean values and error bars represent standard deviation. The vertical scatter plots for these graphs represent the value of mean similarity and stereotypy values per bird in all graphs. Values for (**a**) linearity, (**b**) consistency and (**c**) stereotypy were higher for controls than for naltrindole-treated birds, with the largest difference observed in linearity

when songs of control and treated birds, or siblings versus their fathers' songs were compared.

Blocking δ-ORs during the sensitive period of learning leads to an increase in DARPP-32-positive neurons within Area X

We found no significant differences in the estimated volumes of LMAN, Area X, HVC and RA in control and treated birds (Fig S3a-f). We quantified the number of DARPP-32-positive neurons within different song control nuclei and found that whereas primary vocal control areas LMANcore and RA lack DARPP-32-positive neurons, these neurons are sparse in HVC [63]. The only 'core' AFP region showing robust DARRP-32 expression was Area X [63]. Furthermore, the number of DARPP-32-positive neurons within Area X of treated birds was higher than those of their control siblings ($t = 2.192$, $p = 0.042$; Fig. 6a, b, and e). The difference in the number of DARPP-32-positive neurons in LMANshell of treated birds was not significantly different from that of controls (Fig. 6c, d, and f).

Since DARPP-32-positive neurons increased in number specifically in Area X following naltrindole administration, we decided to study whether there were changes in synapses in this region. The pre- and post-synaptic markers synaptotagmin and PSD-95, respectively, (Fig. 7a) were visualized using immunohistochemistry and used to estimate the number of synapses in the DARPP-32-rich Area X. The synapses were counted as the number of points of contact of synaptotagmin (green) and PSD-95 (red) in zebra finch brain sections, as described in [64] (see Methods; Fig. 7b, c). The total number of synaptic contacts were significantly higher in Area X of naltrindole-treated birds than in controls ($t = 2.241$, $p = 0.038$; Fig. 7d). Comparisons between the average values of puncta in individual sibling pairs showed that there were a greater number of synapses in the Area X of treated birds within each experimental set (Fig. 7e).

Discussion

Our results indicate, for the first time, that blocking δ-OR signaling for a short period (10 days) at the beginning of the sensorimotor phase of vocal learning in zebra finches resulted in poor song quality at adulthood. Whereas there were no significant changes in terms of number of motifs/bout directed towards females, the acoustic features of songs were affected by naltrindole administration during the sensitive period. Specifically, there were changes in spectral features of songs including significant decreases in pitch, frequency modulation and mean frequency sung by treated birds versus their siblings (vehicle-controls) at adulthood. Furthermore, the songs of treated bird were simpler, with fewer FM syllables versus harmonic stack syllables. Blocking δ-ORs during the

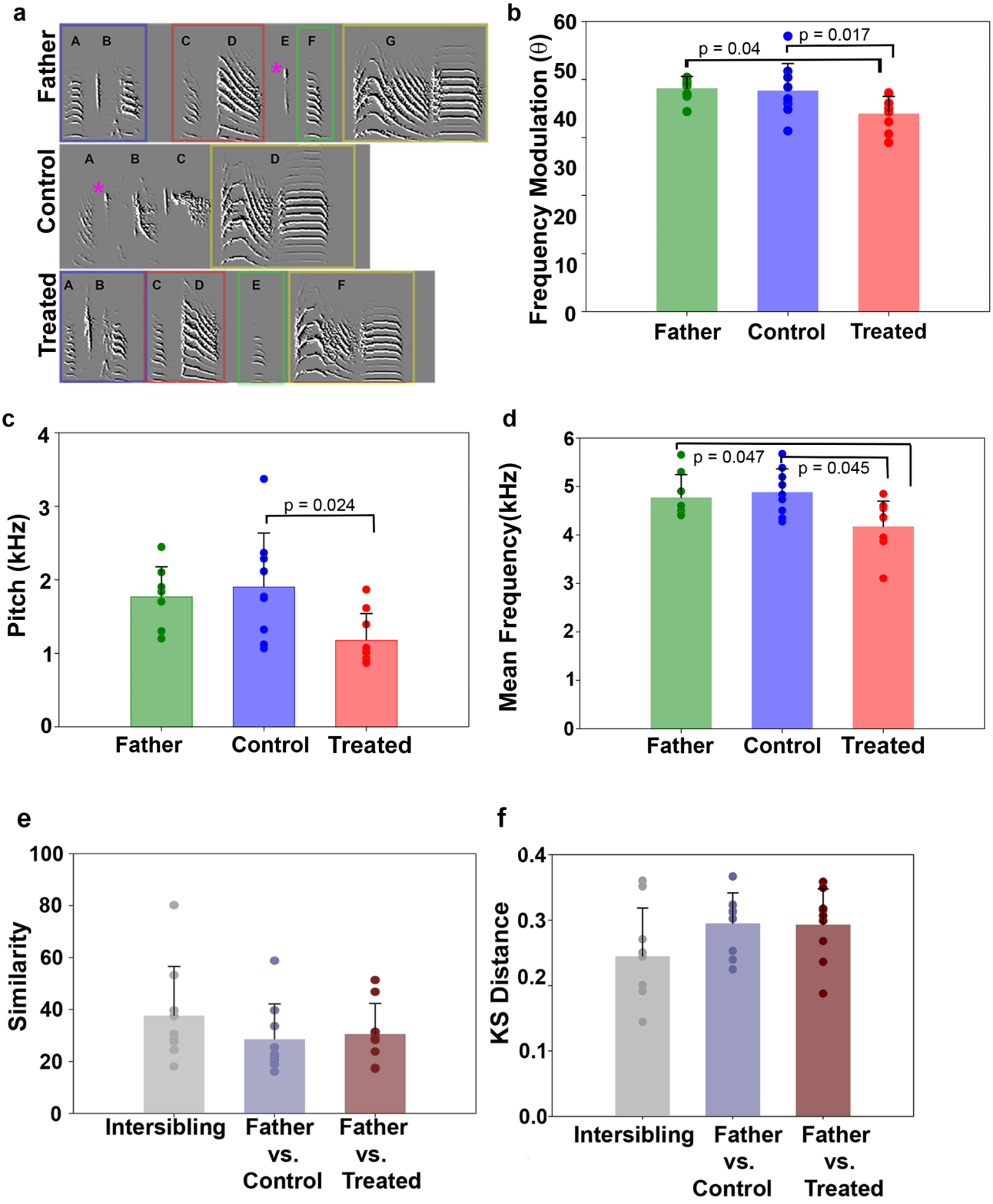

Fig. 5 Comparison of spectral and temporal features of the tutor's songs to those in the songs of their offspring. (**a**) Sonograms demonstrating similarities in syllables (outlined) sung by one set of a tutor and his control and treated offspring. The asterisk represents a note only copied by the control bird. Bars graphs represent mean values and error bars represent standard deviation. The vertical scatter plots for these graphs represent the value of mean similarity and stereotypy values per bird in (**b-f**). The (**b**) frequency modulation, (**b**) pitch, and (**c**) mean frequency of the songs of control birds were similar to that of their fathers and significantly greater than that of the treated siblings. (**e**) Although these differences were not significant, overall similarity in the songs was the highest between control and treated siblings, who also had the shortest (**f**) Kolmogorov-Smirnov (KS) distance

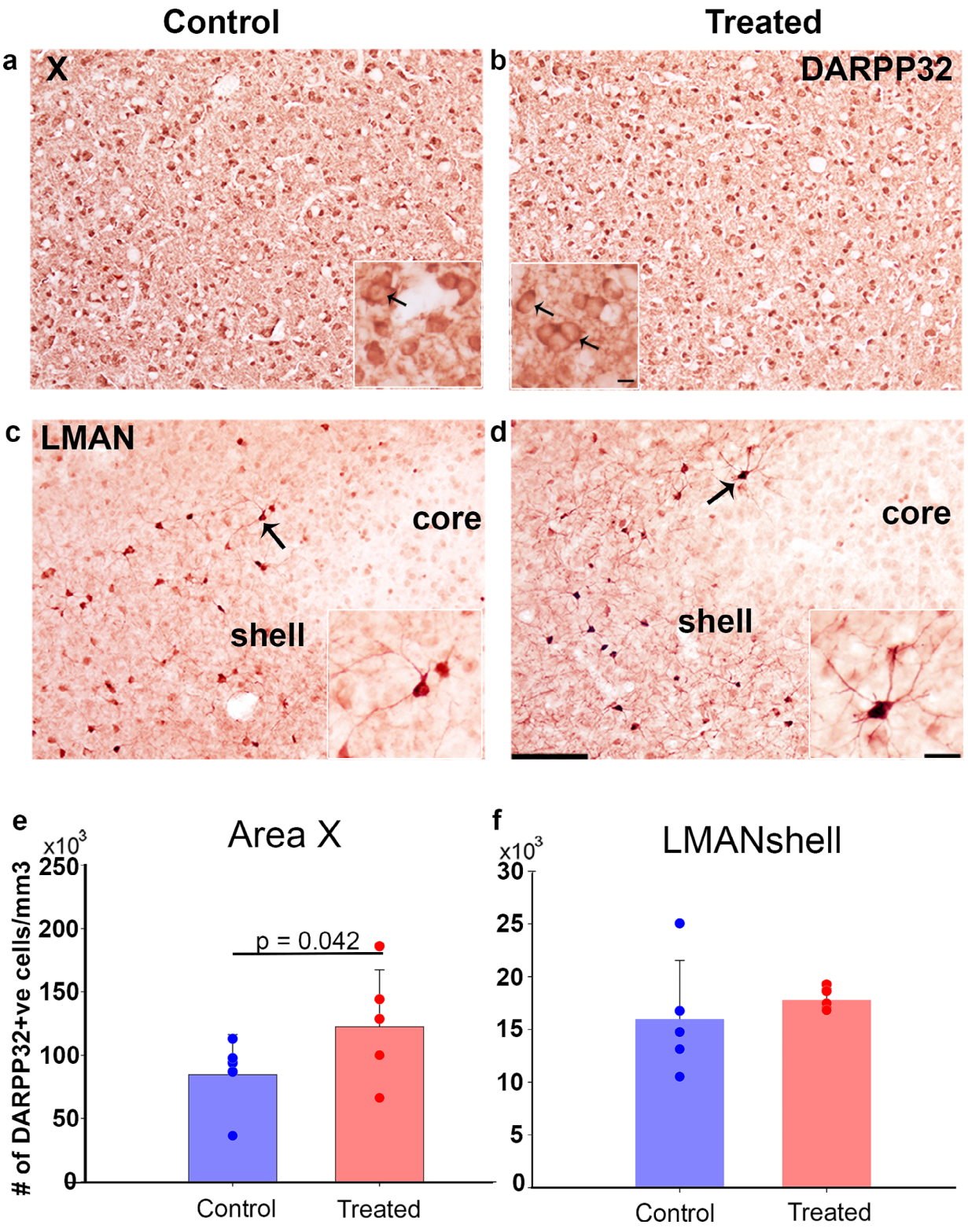

Fig. 6 Quantification of DARPP-32-positive neurons in the basal ganglia song control nucleus Area X. Well-labelled DARPP-32-positive cells were present in Area X and LMANshell of both control (**a**, **c**) and treated (**b**, **d**) birds, respectively. Insets in these figures show DARPP-32-positive neurons at high magnification (Scale bar, 100 µm; for insets, 10 µm). For Fig. 6e and f, bar graphs represent mean values, and error bars represent standard deviation; the scatter plot represents the average number of DARPP-32-positive cells per bird. (**e**) Administration of the δ-OR antagonist naltrindole for a 10-day period during the sensitive period of learning resulted in a significant increase in the number of DARPP32-positive medium spiny neurons in Area X. (**f**) Although the number of DARPP32-positive cells were also higher in the LMANshell of treated birds, this difference was not significant

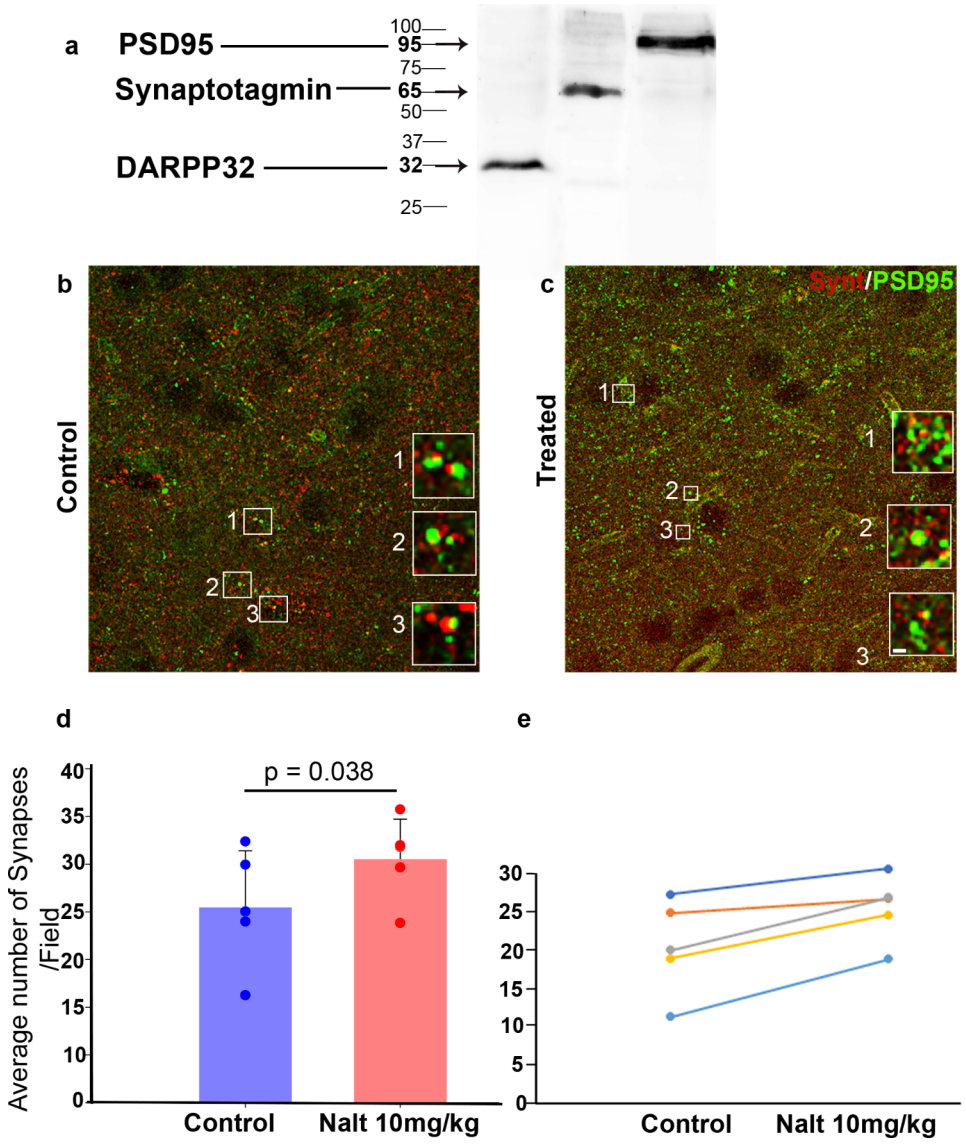

Fig. 7 (**a**) Single bands were obtained in western blots performed using zebra finch brain lysate for PSD-95 at 95kD, Synaptotagmin at 65kD and DARPP-32 at 32kD using specific antibodies (anti-PSD-95, ab9708, Abcam, USA; anti-synaptotagmin, MAB5200, MERCK Millipore; anti-DARPP32, ab40801, Abcam, USA). The synaptic markers were used to label synapses in (**b**) control and (**c**) treated birds, respectively. Insets show a magnified view of the synaptic contacts (yellow). (**d**) There was a significant increase in the number of possibly excitatory synapses within the song control nucleus Area X of treated birds during development, suggesting that altering δ-OR signaling can cause long term changes in synaptic connectivity within the basal ganglia. Scale bar, 10 µm. Bar graphs represent mean values and error bars represent the standard deviation. Each dot in the scatter plot represents average puncta values per field per bird. (**e**) Comparisons of the number of synapses between siblings showed that a higher number of synapses were observed in Area X of the naltrindole-treated birds versus controls in each set

sensitive period also led to changes in the note sequence, leading to a decrease in linearity and stereotypy of songs sung in adulthood. Interestingly, the changes in behavior could be linked to changes in the number of DARPP-32-positive neurons and synapses in Area X in naltrindole-treated birds.

Naltrindole-treated birds develop a simple song structure with a decrease in spectral properties compared with their tutors and control siblings

We observed that injections of the δ-OR antagonist naltrindole during the sensitive period for vocal learning resulted in a low-pitched stereotyped song. Earlier studies have shown that dopaminergic inputs to the basal ganglia nucleus Area X are required for learning pitch [66, 67]. Zebra finches were trained to shift their pitch to a targeted threshold by white noise-induced negative

reinforcement. Birds lacking the VTA → Area X dopaminergic projections were unable to achieve the targeted pitch [66]. Possibly, pitch learning occurs via reinforcing error signals generated by VTA dopaminergic projections to Area X, which are responsible for internal evaluation of vocal learning [66]. Since Area X also expresses high levels of δ-ORs [53], the endogenous opioid system might also be involved in pitch learning and frequency modulation of syllables. This is further supported by anatomical studies in mammals, showing δ-OR and dopaminergic receptor co-expression on striatal neurons [28], which might influence each other's signaling pathways [33]. Furthermore, both μ- and δ-ORs regulate downstream dopaminergic targets such as DARPP-32 [68]. These studies suggest that interaction between dopamine and opioid receptors within the striatum might modulate pitch learning in young zebra finches.

Our results also demonstrated that the songs of naltrindole-treated birds were simpler as they mostly incorporated harmonic-stack types of syllables resembling call-like notes [69]. The presence of a greater number of call-like notes is observed in zebra finches exposed to white noise during the sensitive period of song learning [70]. We further observed that the few FM syllables present in treated birds' songs were poorly developed with shorter duration, low pitch, and low frequency modulation than those produced by controls. Additionally, the songs of treated birds differed from their fathers in terms of spectral quality, wherein the songs of treated birds had significantly lower pitch, mean frequency and FM. These results suggest that pharmacological blocking opioid receptors during the sensitive period may interfere with the birds' ability to learn and/or produce intricate frequency modulations. Alternatively, it is possible that naltrindole-treated birds may have practiced their songs to a lesser extent between the 45d-80d period, following injections, leading to deficits in their adult songs.

The behavioral deficits observed in naltrindole-treated birds were similar to those resulting from Area X lesions in juvenile birds and were accompanied by an increase in DARPP-32-positive neurons, which is also seen following lesions of Area X (see below [71]),. Juvenile zebra finches with Area X lesions during the sensitive period (31dph) had poor syllable structure and motif stability, which was similar to the behavioral deficits in naltrindole-treated birds in our study [72]. Similarly, neurotoxic lesions of Area X resulted in a decrease in syllable duration and an increase in the repetition of syllables akin to stuttering [50]. The similarity between impact of Area X lesions and our results on learnt song suggests that administration of the δ-OR antagonist naltrindole between 35d and 45d during the sensitive period for vocal learning may have had a greater effect on the AFP in general and Area X in particular. This is because at this time, juvenile birds sing only subsong, which is modulated by LMAN (a component of the AFP) and is not eliminated by HVC lesions [73]. Furthermore, whereas axons from LMAN-core have synapsed onto RA neurons by 15d post-hatch, HVC axons begin to synapse onto RA neurons only at ~30d [74, 75]. It is therefore possible that changes in song learning and vocalization stemming from blocking δ-ORs between 35 and 45d post-hatch would depend to a greater extent on components of the AFP rather than the VMP.

Changes in neural architecture in juvenile zebra finches treated with naltrindole

Other than the behavioral effects seen after Area X lesions, Kubikova et al. (2014) [71] also showed that lesion sites within Area X had increased recruitment of newly generated DARPP-32-positive medium spiny neurons (MSNs). These neurons receive dopaminergic input from midbrain VTA-SNc and glutamatergic input from the pallium [76, 77]. Interestingly, Area X is the only song control region within the 'core' AFP which is enriched with DARPP-32-positive neurons [63]. Our results, showing that a larger number of DARPP-32 neurons in the basal ganglia of naltrindole-treated birds, are partly similar to the effects obtained after neurotoxic lesions in Area X [71]. It is possible that the addition of DARPP-32 neurons in Area X may represent a repair mechanism induced after naltrindole treatment. Since it takes about 42 days for DARPP-32 neurons to functionally incorporate into Area X [78], any changes in their numbers occurring during the sensitive period may have manifested before song crystallization. Furthermore, DARPP-32 neurons are constantly added but not replaced in the striatum [78]. Therefore, it was possible to quantify neural changes within Area X four months after naltrindole treatment.

Besides the increase in dopaminoceptive neurons (MSNs), we also found an increase in potentially excitatory synapses within Area X, which received excitatory input from both HVC and LMAN (see [39] for review), following naltrindole administration during the sensitive period. These findings may have resulted from the increase in the postsynaptic target, that is, MSNs, in treated birds versus controls. Whereas LMAN induces variability in spectral features and the sequence of syllables [79], Area X modulates fundamental frequency at the syllable level [80]. An increase in vocal variability due to an imbalance in inhibitory control provided by MSNs might be the reason underlying the loss of song stereotypy in naltrindole-treated birds. Taken together, these findings suggest that changes in opioid modulation may lead to changes in other parts of the song control circuitry and need further investigation.

Caveats

We understand that systemic administration of naltrindole is an obvious limitation of this study. Additionally, the high sequence homology between μ-, δ- and κ-ORs [81] and the presence of opioid heterodimers [82] also suggest that the overall behavioral and neural changes reported here may have been caused by a cumulative effect of blocking different OR subtypes. Further studies are needed to evaluate the individual effects of these receptors on the development of song structure as well as the effects of naltrindole treatment on different song control nuclei. Lastly, we assumed that the previously tested dose of 10 mg/kg [15, 17] for OR antagonists would result in a constant and chronic antagonism of δ-ORs for 10 days. However, the lack of data of the affinity of avian δ-ORs for naltrindole prevented actual quantification of the efficacy of δ-OR antagonism. Despite these limitations, this is one of the few studies that aims to study the role of opioids in vocal learning.

Conclusions

In conclusion, we report that δ-ORs may modulate the development of song structure in young zebra finches. Blocking δ-OR signaling for a ten-day period during the sensorimotor phase of learning in juvenile male zebra finches without perturbing their social environment resulted in abnormal acoustic features, loss of stereotypy in the crystallized song and long-term changes in the neural circuitry underlying song learning. We predict that an imbalance in inhibition within the striatum might affect neuronal activation in the AFP, resulting in poor song structure. These results taken together with future research on the identification of precise neural pathways and neurochemical changes associated with δ-OR signaling, in relation to vocal behavior, will contribute to a better understanding of how δ-ORs influence higher cognitive functions.

Abbreviations

AFP	Anterior forebrain pathway
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa
δ-ORs	Delta opioid receptors
κ-ORs	Kappa opioid receptors
μ-ORs	Mu opioid receptors
DLM	Dorsolateral nucleus of the medial thalamus
dph	Days post hatch
HVC	Abbreviation used as a proper name
LMAN	Lateral magnocellular nucleus of the anterior nidopallium
LMANcore	Magnocellular core of LMAN
LMANshell	Parvicellular shell of LMAN
MSN	Medium spiny neuron
RA	Robust nucleus of the arcopallium
VMP	Vocal motor pathway
VTA	Ventral tegmental area
VTA-SNc	Ventral tegmental area-substantia nigra complex

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12868-025-00927-x.

Supplementary Material 1

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Author contributions

UAS performed the experiments and behavioral studies, histological studies, confocal microscopy and puncta analysis, and analyzed the data. SI designed the experiments and supervised the study. UAS and SI interpreted the data, and drafted the manuscript. Both authors contributed to the scientific discussions and approved the final manuscript for publication.

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Data availability

Data is provided within the manuscript and supplementary information files. Original files used to generate the data in the manuscript are available in the Dryad Data Repository: DOI: https://doi.org/10.5061/dryad.x69p8czsh; Reviewer URL: https://datadryad.org/stash/share/67cRHIp3o2oCepMVIp7UvfjzcJOxRkQomVert5BaJye.

Declarations

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Ethics Committee at the National Brain Research Centre, Manesar, in accordance with the guidelines laid down by the Committee for the Control and Supervision of Experiments on Animals (CCSEA), India (protocol number: NBRC/IAEC/2013/85). All zebra finches used in our experiments were obtained from the Animal House at NBRC, Manesar.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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