Delta Opioid Receptors affect Qualitative Features of Song during Vocal Learning in Zebra Finches.

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Table S1: Results of Two-way RM ANOVA to test the effect of age and treatment on mean values of syllable features

Category	**Variable**	**F statistic**	**p-value**	**Degrees of freedom**	**Post-hoc analysis (Bonferroni)**
Pitch	Age	F = 0.187	0.943		
	Treatment	F = 51.45	<0.001	1	t = 7.18; p<0.001
Mean Frequency	Age	F = 0.218	0.927		
	Treatment	F = 64.812	<0.001	1	t = 8.051; p<0.001
Frequency Modulation	Age	F = 0.824	0.518		
	Treatment	F = 38.272	<0.001	1	t = 6.186; p<0.001

1. Dose-Response curve for Naltrindole in adult male zebra finches:

Animals

Adult male zebra finches (>100days; n =28) were used for this experiment after screening them for the number and quality of songs. Birds that sang an average of 25 songs in 30 minutes and had at least three syllables in their songs were selected for this study. A cage (12 X 15 X 11 inches) housing a single male zebra finch was placed adjacent to another cage containing a set of three female zebra finches. Songs directed towards the females were recorded using a Sony Handycam (DCR-SR67E E37) and a microphone (Sennheiser e614, M-track quad audio device) connected to Sound Analysis Pro software version 1.02).

Drug administration

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 (1mg/ml). The drug dose was calculated according to the weight of the birds (average, 12.01gm) and the stock was diluted to 100 µl volume for injections.

Seven sets of male zebra finches were used for these experiments. Each set comprised of four birds which were administered intramuscular injections of 100µl of 0.9% saline on the first two days of the experiment followed by the δ-OR antagonist naltrindole at a dose of 1, 2.5, and 5mg/kg, once daily for the next 4 days, respectively. One set of birds acted as a general control for the experiment and received intramuscular injections of 0.9% saline on all 6 days of our experimental protocol.

Behavioral recordings

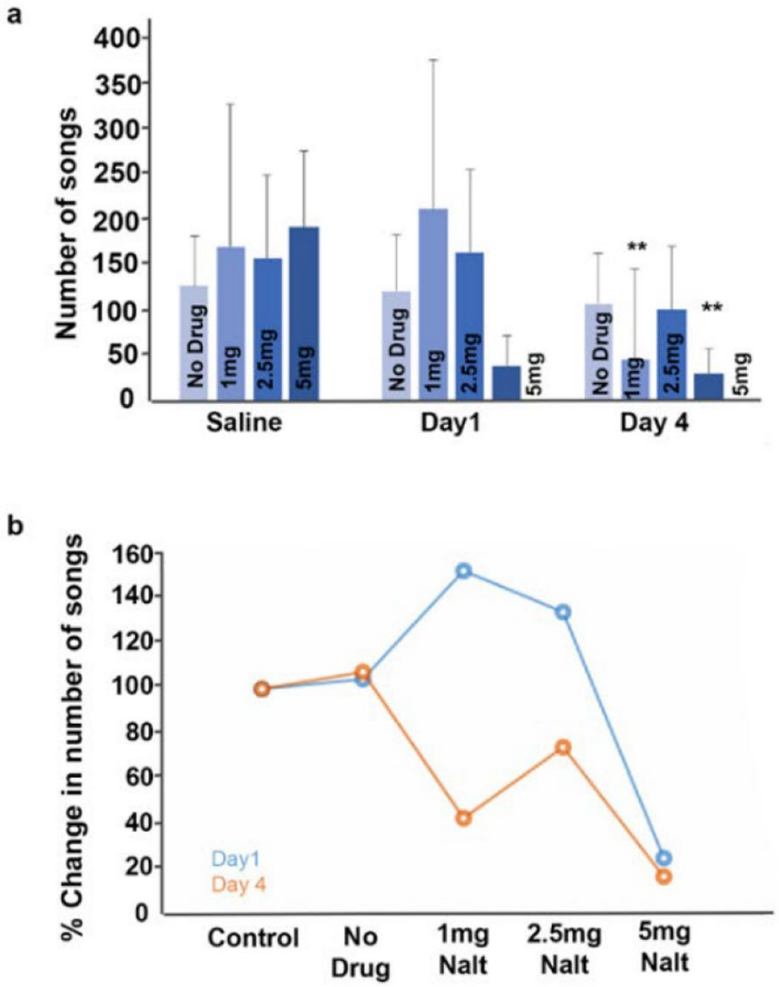
Behavioral recordings were performed in a recording cage of dimensions 12 X 15 X 11 inches placed in a sound-attenuated chamber. Female birds were housed in a separate cage, which was placed next to the recording cage. The Goldwave (Ver.- 5.10 Goldwave Inc.) and Audacity (Ver.- 3.14-beta) software were used to record songs and calls. Female-directed songs were recorded for 45 minutes after administration of either the drug or saline. All recordings were performed during the first half of the day and began after a ten-minute gap post injection which was to allow the birds to recuperate from the stress of handling and drug /saline administration. Besides recording their songs, a video camera (Sony Handycam; DCR-SR67E E37) was used to record stereotyped behavior and movement. The video recordings were also used to assess whether the birds produced female-directed songs. All male birds selected for these experiments were housed together in a holding cage before and after the recordings.

Statistical Analysis:

We calculated the number of songs for saline and naltrindole-treated adult birds on the first and fourth day of saline or naltrindole administration. Song recordings on the first two days of saline injections also served as within-subject controls to test the difference in the spectral and temporal features of song. We used average values from the first two days of the control recordings to compare with the average values of spectro-temporal features from the first and fourth day of drug administration for each bird. The repeated measures analysis of variance (RM ANOVA; SigmaPlot 12; Systat Software Inc., USA) was used to test whether there were differences in the performance of various behaviors across different days of naltrindole

treatment. The Holm-Šidák method was used post-hoc to test for significance in multiple comparisons.

Fig. S1

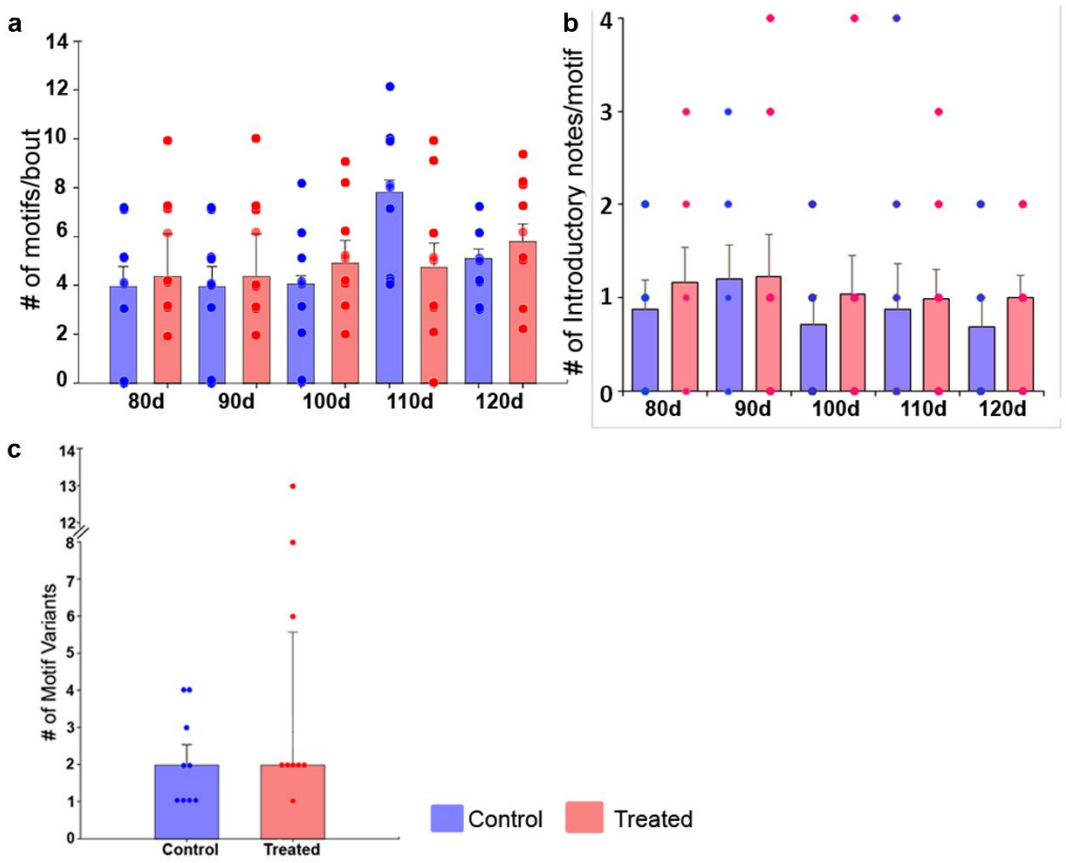


Results

We found that there was a significant decrease in the number of songs on the fourth day following administration of the 1mg/kg dose of naltrindole between the treatment groups [$F_{(2, 5)} = 5.478$, $P = 0.025$]. Although there was a 52% increase in the number of songs on the first day of the 1mg/kg dose, it was followed by a significant decrease (58%) on the fourth day of drug administration [$F_{(2, 5)} = 5.224$, $P = 0.028$]. There was a steep decrease in the number of songs across different treatments for the 5mg/kg dose [$F_{(2, 7)} = 22.993$, $P < 0.001$], translating into an 87% decrease in the number of songs produced after naltrindole administration. Pairwise comparisons revealed that the number of songs decreased both on the first ($P < 0.001$; $t = 6.018$) and fourth ($P < 0.001$; $t = 5.716$) day of administering 5mg/kg dose of naltrindole compared with controls. We did not find any difference in the amount of singing after the administration of the 2.5mg/kg dose of naltrindole [$F_{(2, 5)} = 1.857$, $P = 0.206$] (**Fig. S1 a** and **b)**.

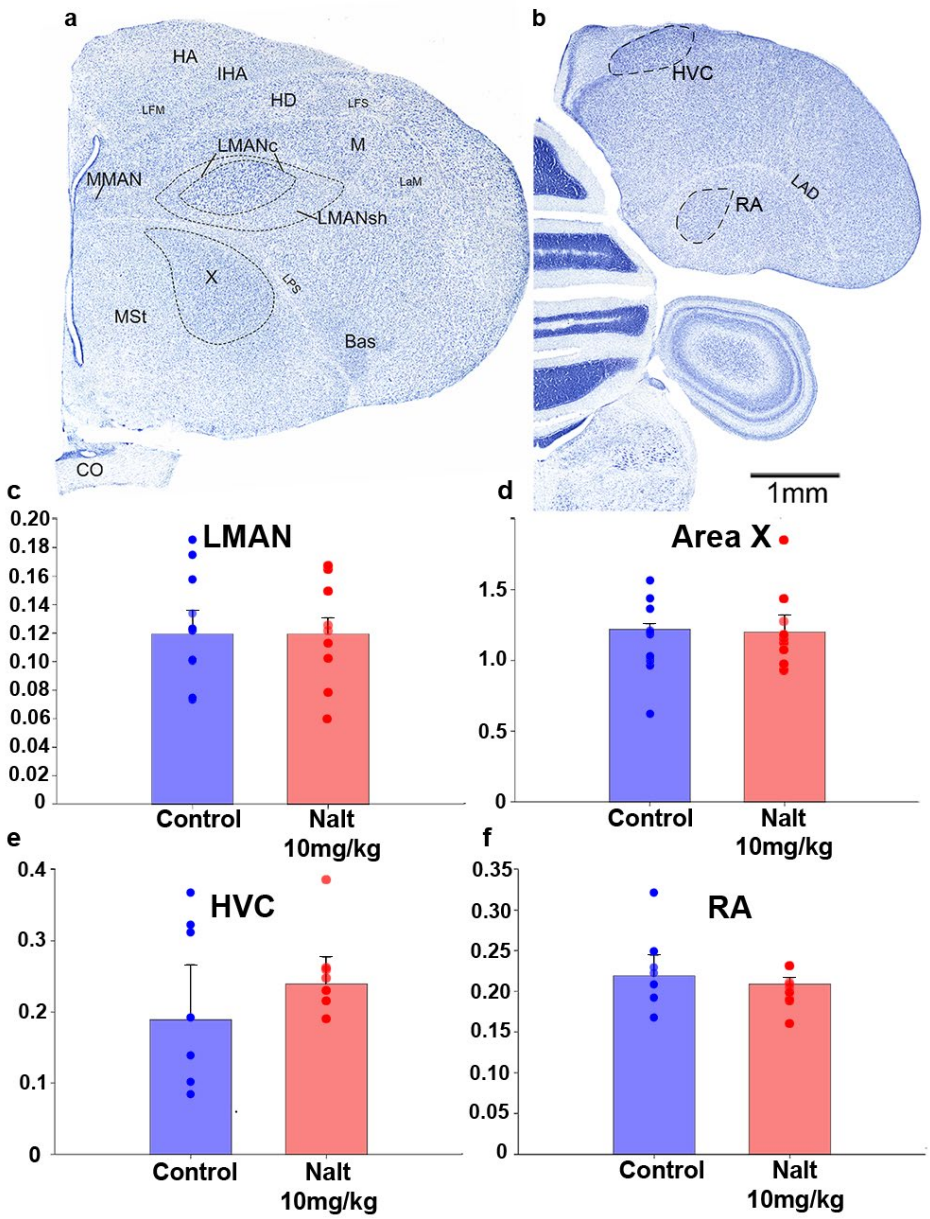
2. Naltrindole treatment was not associated with a decrease in the number of songs

We counted the number of introductory notes which are variable numbers of short vocalizations sung just before the bout and represent motor preparation for the production of learned song . The number of introductory notes per motif was a ratio of the total number of introductory notes and the total number of motifs produced during a recording. Similarly, for the number of motifs, we used the ratio of the number of songs and the total number of bouts produced by the bird to represent the difference between the number of songs produced by control and treated birds. Furthermore, the song repertoire was assessed by calculating the number of motif variants produced by the birds. Song variants that occurred at a frequency of about 10% within the bout were quantified. Similarly, we calculated the ratio of the number of songs and the total number of bouts to represent the difference between the number of songs produced by control and treated birds. Furthermore, the song repertoire was assessed by calculating the number of motif variants produced by the birds. Song variants that occurred at a frequency of about 10% within the bout were quantified.



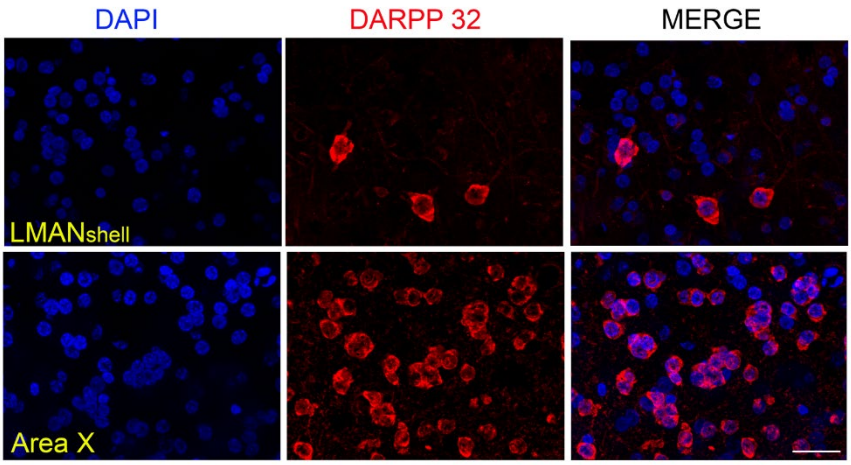
Supplementary Figure (S2): The number of songs, introductory notes and motif repertoire of control and naltrindole-treated birds. Bar graphs represent mean values and the error bars represent standard deviation. (**a**) We observed no differences in the number of songs or the (**b**) number of introductory notes produced by control and treated birds. (**c**) Although the number of motif variants were greater in the treated birds versus controls, this difference was not significant.

3. Volumetric analysis of song control areas



Supplementary Figure 3 (S3): Nissl-stained song control areas and volumetric measurements. All song control nuclei were outlined using an Olympus microscope (BX-51) with the help of the Stereoinvestigator software (version 10, Microbrightfield, USA). The Cavalieri estimator probe was used to estimate the volume of each nucleus in mm3. Volume estimation was performed on the first Nissl-stained section in a series of four sections, using a grid spacing of 10µm at a magnification of 40X. (**a, b**) The major song control nuclei were outlined using Nissl-stained coronal sections of the zebra finch brain. These areas included the pallial song control nuclei LMAN, HVC and RA and the basal ganglia nucleus Area X. There was no significant difference in the total volumes of (**c**) LMAN, (**d**) Area X, (**e**) HVC and (**f**) RA across controls and naltrindole-treated birds. Y-axis represents mean volumes in mm^3, error bars represent standard deviation.

4. Staining for DARPP-32 in LMANshell and Area X:



Supplementary Figure 4 (S4): Sections from the anterior forebrain were mounted on gelatin-coated slides and were subjected to antigen retrieval using the antigen unmasking solution (citrate buffer, pH 6.0, H-3300, Vector Laboratories, Burlingame, CA, USA) at 80ºC for 30 min. This was followed by blocking with 5% Normal Goat Serum (NGS; S-1000, Vector Laboratories, Burlingame, CA, USA) and 1% BSA for 1 h and incubation in a solution containing Anti-DARPP-32 (ab40801, abcam; 1:500) for 12 h at 4 ºC. The slides were rinsed with PBS, after which they were incubated in a secondary antibody solution (1:500) containing Goat Anti-Rabbit IgG Alexa Fluor 594 (A-11008, Invitrogen) for 2 h. Sections were rinsed in PBS and mounted with VECTASHIELD Anti-fade mounting medium containing DAPI (H-1200, Vector Labs). Cytoplasmic staining for DARPP-32 can be clearly observed in neurons in both LMANshell and Area X, and nuclei are labelled with DAPI in the merged image. Scale bar, 20µm.