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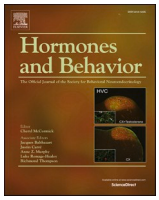

Chronic intranasal oxytocin increases acoustic eavesdropping and adult neurogenesis

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ABSTRACT

Social information gathering is a complex process influenced by neuroendocrine-modulated neural plasticity. Oxytocin (OXT) is a key regulator of social decision-making processes such as information gathering, as it contextually modulates social salience and can induce long-term structural plasticity, including neurogenesis. Understanding the link between OXT-induced plasticity and communicative awareness is crucial, particularly because OXT is being considered for treatment of social pathologies. We investigated the role of chronic OXT-dependent plasticity in attention to novel social information by manipulating the duration of time following cessation of intranasal treatment to allow for the functional integration of adult-born neurons resulting from OXT treatment. Following a 3-week delay, chronic intranasal OXT (IN-OXT) increased approach behavior of both female and male mice towards aggressive vocal playbacks of two unseen novel conspecifics, while no effect was observed after a 3-day delay. Immature neurons increased in the ventral hippocampus of females and males treated with chronic IN-OXT after the 3-week delay, indicating a potential association between ventral hippocampal neurogenesis and approach/acoustic eavesdropping. The less the mouse approached, the higher the level of neurogenesis. Contrary to expectations, the correlation between ventral hippocampal neurogenesis and approach behavior was not affected by IN-OXT, suggesting that other plasticity mechanisms underlie the long-term effects of chronic OXT on social approach. Furthermore, we found a negative correlation between ventral hippocampal neurogenesis and freezing behavior. Overall, our results demonstrate that chronic IN-OXT-induced long-term plasticity can influence approach to vocal information and we further reinforced the link between neurogenesis and anxiety.

1. Introduction

The bidirectional transmission of information from one individual to another defines social species. This communication requires an individual to not only produce communicative signals, but to in turn understand the social information it receives. In social mammals, the neuropeptide oxytocin (OXT) is important for processing such communicative information (Marlin et al., 2015; Tang et al., 2020). For example, maternal pup retrievals in mice rely on OXT-enhanced auditory cortical responses to pup calls (Marlin et al., 2015). Conversely, OXT antagonists impair recognition of communication cues and disrupt neural response to chemical communication in adult male mice (Samuelsen and Meredith, 2011). In humans, heightened endogenous OXT release is associated with increased synchrony of social interactions between test partners, and exogenous OXT increases facial and vocal responses to social-emotional cues (Barchi-Ferreira and Osório, 2021; Spengler et al., 2017). In an exploratory analysis OXT improved performance on the "reading the mind in the eyes test" negative emotion subscale (Melby et al., 2022) further indicating that OXT signaling may play a role in social-emotional recognition. The OXT-dependent processing of communication extends to agonistic social interactions as well, with intranasal OXT reducing reactive aggression to social provocation in adult men (Zhu et al., 2019). Intranasal OXT (IN-OXT) can also reduce the avoidance of emotionally evocative social stimuli (Harari-Dahan and Bernstein, 2017). Taken together, a large body of research suggests that acute OXT increases the perception and response to both positive and negative social cues seen across mammals (Winterton et al., 2021).

Prevailing theories of the social effects of OXT indicate that acute OXT may therefore increase social salience (Shamay-Tsoory and Abu-Akel, 2016) and approach-related social behaviors (Kemp and Guastella, 2011). Accordingly, exogenous OXT is being considered as a

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therapeutic target for socio-affective disorders due in part to its ability to context-dependently promote social processing and communication. For example, IN-OXT led to increased willingness to act socially and engage in social coordination for subjects with autism spectrum disorder (Andari et al., 2010; Auyeung et al., 2015). IN-OXT also enhanced amygdala-frontal resting state functional connectivity in patients with generalized social anxiety disorder (Dodhia et al., 2014) and attention to the mouth regions of faces in depressed men (Boyle et al., 2022). As a repeated-dose regimen of IN-OXT is more clinically realistic and representative of long-term treatment than a single-dose regimen (Horta et al., 2020), studies that investigate the socio-behavioral and neurobiological changes associated with chronic OXT administration are key to understanding the clinical applicability of OXT treatment. However, such studies are performed less frequently than acute studies, and have produced conflicting results on affiliative behavior. It is likely that chronic OXT effects are dependent on a multitude of factors, including life-stage-, sex-, species-, and social and environmental context- specific features (Arias del Razo et al., 2020), and may result in either approach or avoidance of social stimuli.

Additionally, the timing of testing following the cessation of chronic treatment may reveal differential effects of chronic IN-OXT, suggesting that delayed-onset long-term plasticity may play a role. For example, chronic IN-OXT reduces social defeat stress-induced social avoidance in female prairie voles the day following the cessation of treatment (Hale et al., 2021). In titi monkeys, chronic IN-OXT increased tail-twining in pairs, and males displayed greater affiliation in adulthood persistently after chronic juvenile/pubertal treatment (Razo et al., 2022). However, IN-OXT also impairs pair-bond formation in male prairie voles when given during development (Bales et al., 2013). While chronic intranasal studies investigating social behavioral dynamics are on the rise, little is known about how chronic IN-OXT impacts social information gathering behaviors such as acoustic eavesdropping and what underlying long-term neuroplasticity may be involved. Moreover, few studies have directly compared the effects of adult-administered chronic IN-OXT at short and long time points following treatment cessation.

For species that exhibit both affiliative and aggressive behavior, social information gathering is the first step in mounting an appropriate response for the social context. Monitoring vocal exchanges between other conspecifics allows animals to gather social information (Beecher et al., 2007; Peake et al., 2002). This form of social information gathering, known as acoustic eavesdropping, can be a relatively safe way for individuals to determine the motivations and capabilities of rivals (Bernal and Page, 2023). For example, great tits eavesdrop on conspecific altercations in their proximity to gain information about an unfamiliar intruder (Peake et al., 2002) and territorial adult southern white rhino males eavesdrop on trespassing rival male contact and courtship calls to determine dominance status (Cinková and Shrader, 2020). We speculate that OXT influences the process of collecting information before social valence is decided and a decision is made for how to engage.

The California mouse is an ideal model with which to study the effects of chronic IN-OXT on acoustic eavesdropping as it is a monogamous rodent that relies heavily on vocal communication throughout affiliation and aggression. A rich ultrasonic vocal repertoire is engaged during pair-bond formation (Pultorak et al., 2018), and maintenance (Pultorak et al., 2017), as well as in parental behavior (Guoynes and Marler, 2021, 2022). Other behaviors such as coordinated territoriality (Rieger et al., 2021) and territorial aggression (Rieger et al., 2019) also involve production and attunement to ultrasonic vocalizations. In previous studies in which we exposed California mice to playbacks of aggressive vocalizations we observed no response vocalizations when mice were tested individually in a neutral arena (Monari et al., 2021; Rieger et al., 2021), suggesting vocal attention but not engagement. Moreover, these mice had never before established a territory at any location and were tested at a developmental timepoint where they would be expected to explore the social environment to establish a territory (Zhao et al., 2020). For California mice, separation from the natal group is a critical time of social information gathering, as individuals face the challenge of interacting with novel conspecifics, likely for the first time and with relatively high frequency. Movement out of a natal territory context has been shown to induce neuroplasticity (Zhao et al., 2020). This period of heightened opportunity for eavesdropping is highly dependent on vocal-social signals. Moreover, IN-OXT likely impacts the perception of and response to social-vocal information, as an acute dose drives pair mate approach coordination to stranger vocalizations (Monari et al., 2021). Thus, changes in responses to ultrasonic vocalizations as a result of chronic IN-OXT are of interest in this species (Marler and Monari, 2021), and the effects of chronic OXT on social information gathering are of broad significance across species.

The behavioral effects of chronic IN-OXT treatment in adults likely rely on persistent neuroplasticity mechanisms, as such behavioral effects are observed even after the clearing of exogenous OXT from the system (Arias del Razo et al., 2022). However, the precise neurobiological substrates underlying such behavioral effects remain unclear. Likely, several forms of plasticity, from the receptor (Robinson et al., 2003) to the cellular-structural level (Lin and Hsu, 2018), are involved. Adult hippocampal neurogenesis has emerged as a potential mechanism through which chronic IN-OXT may impact social behavior, as neuronal proliferation is stimulated by OXT signaling (Leuner et al., 2012; Lin et al., 2017) and synaptic maturation of adult-born neurons requires OXT signaling (Pekarek et al., 2022). Additionally, selective ablation of new hippocampal neurons in adulthood results in impaired or otherwise altered social behavior (Cope et al., 2020; Lagace et al., 2010; Opendak et al., 2016). Interestingly, as pair-bonding and sexual behavior increase neurogenesis (Castro et al., 2022) and OXT signaling (Loth and Donaldson, 2021; Waldherr and Neumann, 2007), pair-bonding-related changes in affiliative and aggressive behavior may occur through an OXT-neurogenesis-dependent mechanism. It is clear that timing is important for neurogenesis resulting from chronic OXT administration to emerge, as too short a time window following treatment likely precludes the expression of doublecortin, an immature neuronal marker indicative of adult-born immature neurons integrated into hippocampal circuitry (brain collection immediately following 10 days of treatment (Duarte-Guterman et al., 2020)). Functionally-delayed neuroplasticity is a major area of investigation, for example with affective latencies characteristic of conventional pharmacological treatments of depression (Malhi et al., 2020).

What is less understood is the contribution of delayed functional plasticity to the processing of and response to social information following chronic IN-OXT treatment, and whether such effects are dissociable from shorter-term impacts. Using adult female and male California mice, we tested the hypothesis that chronic IN-OXT administration would have differential effects on acoustic eavesdropping in the form of approach towards playbacks of two unseen conspecifics aggressively communicating to each other depending on whether individuals were tested 3 days after ("short delay") or 3 weeks after ("long delay") the cessation of treatment. We also predicted that the difference in response to the vocal stimuli would correspond to differences in the density of immature neurons in the hippocampus during the time of testing based on the timeframes necessary for neurogenesis to occur.

2. Methods

2.1. Animals

44 adult male and 44 adult female California mice (*Peromyscus californicus*, 3–6 months old) from a laboratory colony (at the University of Wisconsin-Madison, established from individuals captured in the Santa Monica Mountains of California) were housed (2–4 same-sex individuals per cage) in standard cages (48 × 27 × 16 cm, lined with aspen bedding and containing a nestlet). Purina 5015™ mouse chow and water were available ad libitum. Housing was maintained at 20–23 °C on a 14:10 h

light:dark cycle with lights off at 11:00 am CST.

2.2. Ethical statement

Animals were maintained according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. Procedures were approved by the University of Wisconsin-Madison College of Letters and Sciences Institutional Animal Care and Use Committee (Protocol L005447). No animals were injured by any of the behavioral manipulations or assays.

2.3. Intranasal oxytocin (IN-OXT)

To compare the effects of time delay on chronic IN-OXT-stimulated neurogenesis (Fig. 1), animals were randomly assigned to one of two groups receiving either a 0.5 IU/kg dose of IN-OXT (Bachem, Torrance, CA, Prod #: 4016373) dissolved in saline (long delay: 11 males, 11 females, short delay: 11 males, 11 females) or intranasal control saline dose (long delay: 11 males, 11 females, short delay: 11 males, 11 females) once daily for seven days 1–3 h following the onset of the dark cycle. Intranasal administration is a non-invasive route of delivery for OXT, and similar doses induce context-dependent changes in social behavior in California mice (Guoynes and Marler, 2021, 2022, 2023; Monari et al., 2021) and other rodents (Guoynes et al., 2018; Murgatroyd et al., 2016; Steinman et al., 2016). Doses of this magnitude also approximate weight-adjusted doses in human studies (Erdozain and Peñagarikano, 2020). While the mechanism is unclear, IN-OXT reaches the brain in house mice (*Mus musculus*) at behaviorally-relevant concentrations (Quintana et al., 2021; Yamamoto et al., 2019).

IN-OXT delivery was performed in California mice as previously published (Guoynes and Marler, 2021, 2022, 2023; Monari et al., 2021). In brief, males and females received 2.5 μl of solution via application to the nostrils using a blunt needle attached to cannula tubing at the end of a Hamilton syringe. Individual droplets were beaded at the end of the needle, applied to the surface of the nose, and allowed to absorb into the nasal mucosa. Administrations were performed rapidly, each lasting <30 s per animal.

2.4. Playback approach test

We investigated individual male and female California mouse approach behavior towards playbacks of bark calls from two aggressively interacting same sex individuals, as in (Monari et al., 2021; Rieger et al., 2021). Testing occurred in dim red light within the first 3 h following the onset of the dark cycle in Plexiglas cages (90 × 30 × 30 cm) equally divided into three chambers (each 30 × 30 × 30 cm) with centrally located openings (11.5 × 11.5 cm) between chambers to allow for free movement. Speakers (Vifa Dynamic Ultrasound, 1–120 kHz range, Avisoft Bioacoustics, Berlin, Germany) were placed at each end of the cage against a closed mesh gate.

Individual mice were habituated to the 3-chamber playback apparatus for 5 min per day for 3 days, with the 2 min playback test occurring immediately following the third habituation. Mice in the long delay experiment began habituations to the apparatus 18 days following the final intranasal administration, as immature neuron density in the hippocampus of male rats increases after a 21 day delay following chronic intraperitoneal OXT administration (Opendak et al., 2016). In contrast to the long delay condition, mice in the short delay experiment began habituations to the apparatus the day following the final intranasal administration.

For the playback approach test, 2 min playback tracks were played from the speakers at opposite ends of the apparatus, with one speaker playing a bark track and concurrent ambient noise track from the second speaker. Video was recorded throughout the 2 min approach test and was later analyzed for freezing, vigilance, time spent in the chamber closest to the bark speaker ("approach chamber") as an approach score, and time spent in the chamber closest to the ambient noise speaker ("avoid chamber") as an avoidance score as in previous studies (Monari et al., 2021; Rieger et al., 2021). A discrimination ratio between time spent in the approach chamber and time spent in the avoid chamber was calculated as (time in approach chamber)/(time in an approach chamber + time in avoid chamber) for each 30s bin of the test to normalize for total exploration time. The discrimination ratio was analyzed over time instead of as a single value in order to assess the impact of habituation to the stimulus.

2.5. Playback stimuli

Playback files were used from previous studies (Monari et al., 2021; Rieger et al., 2021). Briefly, a separate cohort of male and female mice were exclusively used to generate the playback stimuli. Individual mice were placed in a plexiglass cage (50 × 30 × 30) for 24 h under normal

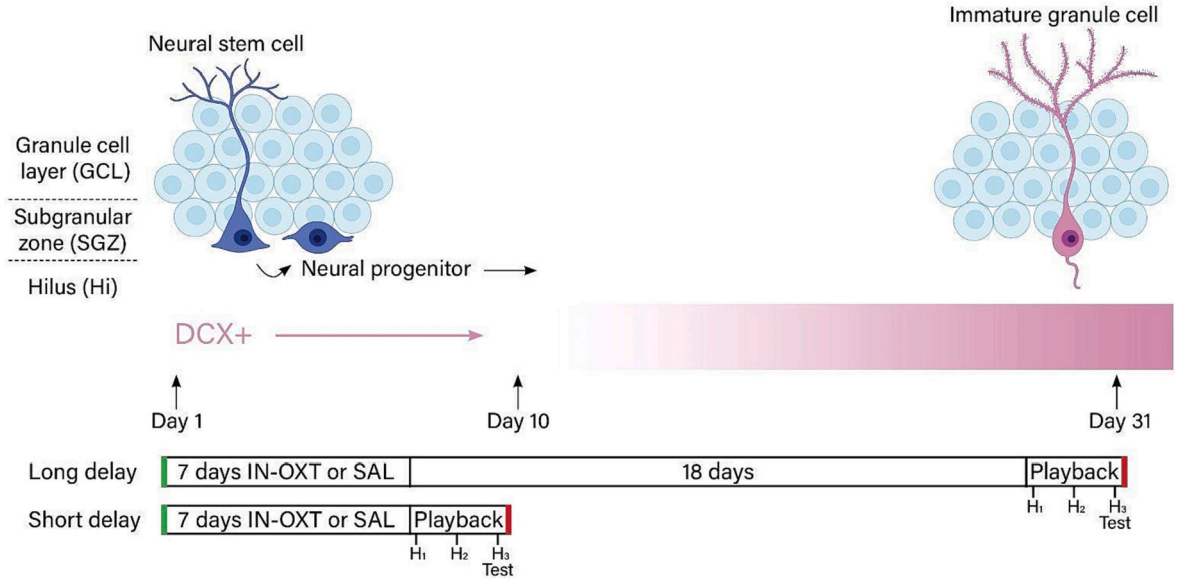

Fig. 1. Experimental timeline aligned with the development of immature neurons in adulthood. Chronic intranasal oxytocin (IN-OXT) may result in increased immature neurons, measured by doublecortin-positive (DCX+) cell density, following a long (21 day) delay.

food and water conditions, as this length of time is sufficient for the formation of territorial residency (Rieger et al., 2019; Rieger and Marler, 2018). Subsequently, residents were introduced to a same-sex intruder for an 8 min period. Each intruder had no previous aggression testing experience and was only used for a single encounter. During the encounter, aggressive barks were recorded using an ultrasonic microphone (Emkay/Knowles FG series, detection range: 10–120 kHz) at a 250 kHz sampling rate and 16-bit resolution, placed 30 cm above the floor of the cage. Barks occur during physically aggressive interactions between individuals of either sex (Rieger et al., 2019; Rieger and Marler, 2018). Following recordings, barks were visibly identified from spectrograms using a 512 fast Fourier transform in Avisoft SASlab pro (Avisoft Bioacoustics, Berlin, Germany). Barks are high-amplitude calls with a negative parabolic shape (Monari et al., 2021). Playback stimuli files were created by selecting only bark calls. Because calls could not be distinguished between the resident and the intruder from the recordings, calls from both individuals were used to construct playback tracks. Barks from female-female pairs are visually indistinguishable from those emitted by male-male pairs with regards to features including frequency modulation and call length (Rieger and Marler, 2018), therefore female-female and male-male playback tracks were randomized during the playback approach test and sex of the bark producer was not used as a factor in analyses in this study. Playback tracks were 2 min in duration and contained 120 ± 5 bark calls. The ambient noise track control was a 2 min recording of the quiet testing room with all lights off and no mice present. We used 8 unique tracks from 8 different sets of individuals and assigned tracks to individuals randomly, with each track used multiple times.

2.6. Immunohistochemistry

The day following the playback test, mice were deeply anesthetized with isoflurane and transcardially perfused with 4 % PFA in PBS. Brains were subsequently fixed for 48 h in 4 % PFA followed by cryoprotection with 30 % sucrose in PBS for 48 h. Unilateral 40 μm-thick coronal sections embedded in OCT were cut using a cryostat (Leica Biosystems). Sections were washed with PBS, blocked with 5 % normal donkey serum, and permeabilized with 0.5 % Tween-20 in PBS for 1 h at room temperature. Rabbit anti-DCX polyclonal primary antibody (Abcam catalog #ab18723) was added for 24 h at room temperature at a dilution of 1:500. After washing, sections were incubated with goat anti-rabbit AlexaFluor-568 (Thermo Fisher Scientific catalog #A-11011) conjugated secondary antibody for 1.5 h in the dark at a dilution of 1:250. Sections were rinsed, mounted onto Suprafrost Plus slides (Thermo Fisher Scientific), dried, and counterstained with Hoechst 33342 (Invitrogen) 1:1000 in water. Slides were then coverslipped using ImmuMount (Thermo Fisher Scientific).

2.7. Cell counts

Immature neuron cell densities were analyzed as previously described (Opendak et al., 2016) throughout the entire rostrocaudal extent of the dorsal and ventral dentate gyrus on every sixth section of a single randomized hemisphere for each mouse (5–6 of each hemisphere per sex, per condition). A BX-60 Olympus microscope assisted by a computer-based system was used to count DCX+ cells and to measure the volume of the granule cell layer for each section. Identification of DCX+ cells was determined using a 63× oil objective and counted by a researcher blind to experimental group. Cell densities were then determined for each section by taking the total number of positively-labeled cells and dividing it by the volume of the granule cell (GC) layer.

2.8. Statistical analysis

Sample numbers were chosen based on previous behavioral studies with similar types of experiments (Cope et al., 2018). Data analyses were performed by an experimenter blind to the experimental group. All statistics were analyzed using R (version 3.6.2) and Rstudio. All datasets are expressed as the mean ± SEM with statistical significance defined as $P < 0.05$. Grubb's outlier test (Tietjen and Moore, 1972) was used to determine outliers, and indicated that no outliers need be removed. Data were analyzed using linear mixed effects models. For cell density models, treatment and sex were used as fixed factors, while individual and slice were used as random factors. For behavior models, treatment, sex, and time bin were used as fixed factors, while individual was used as a random factor. For mixed effects models, F, error df, and *p*-values were calculated from Kenward-Roger approximation. Correlations were Holm-Bonferroni corrected for multiple comparisons. Effect sizes are reported as R^2 for simple linear models and partial ω^2 for linear mixed effects models. The ranges used for determining medium and large effect sizes for R^2 and $\omega^2_{partial}$ are as follows: medium range = 0.09–0.25, large range = 0.25–1.00. Effect sizes for pairwise comparisons are reported as Cohen's *d*.

3. Results

3.1. Chronic intranasal oxytocin increased approach towards aggressive playbacks, with no effect on freezing, after 3-week delay

We investigated the effects of IN-OXT on approach towards a novel vocal playback stimulus three weeks following the cessation of treatment. Chronic IN-OXT increased the amount of time individual females and males spent approaching the bark playbacks, as measured by the discrimination ratio between the playback chamber and the opposite chamber of the three-chamber cage (Fig. 2B; Table S1; $F(1,44) = 21.32$, $p < 0.0001$, $\omega^2_{partial} = 0.41$). There was also an interaction between time and treatment, whereby chronic IN-OXT increased the discrimination ratio over time ($F(1,140) = 7.77$, $p = 0.006$, $\omega^2_{partial} = 0.16$). Pairwise comparisons revealed that chronic IN-OXT increased the discrimination ratio for each 30s bin in females ($p_{30s} = 0.013$, $d = 0.85$; $p_{60s} = 0.029$, $d = 0.73$; $p_{90s} = 0.030$, $d = 0.72$; $p_{120s} < 0.001$, $d = 1.35$) and the last two 30s bins in males ($p_{30s} = 0.77$, $d = 0.07$; $p_{60s} = 0.38$, $d = 0.33$; $p_{90s} < 0.001$, $d = 1.14$; $p_{120s} = 0.011$, $d = 0.79$). There was no interaction with sex nor main effect of sex on the linear relationship of discrimination ratio with time ($F(1,44) = 4.07$, $p = 0.53$, $\omega^2_{partial} = 0.06$).

There was no difference between OXT and saline groups in freezing behavior during the approach test in females or males (Fig. 2C; Table S1; $F(1,44) = 0.10$, $p = 0.759$, $\omega^2_{partial} = 0.03$). Freezing steadily decreased over time for both females and males ($F(1,140) = 20.72$, $p < 0.0001$, $\omega^2_{partial} = -0.19$), confirming habituation to the stimulus. There was also no change in vigilance as a result of treatment (Fig. S1; $F(1,44) = 0.03$, $p = 0.85$, $\omega^2_{partial} = -0.02$). Chronic IN-OXT did not alter freezing in females or males during the pre-test habituation period (Fig. 2D; $F(1,44) = 0.29$, $p = 0.591$, $R^2 = 0.05$).

3.2. Chronic intranasal oxytocin increased adult neurogenesis after a 3-week delay

The rodent hippocampus can produce new neurons into adulthood. Because OXT can increase adult neurogenesis in the adult rodent hippocampus (Leuner et al., 2012), we investigated the effects of chronic IN-OXT both three days (as a control) and three weeks after treatment on immature neuron density in the dentate gyrus in female and male California mice. Examination of the immature neuron marker doublecortin (DCX) revealed that chronic IN-OXT increased the density of immature neurons in female and male ventral, but not dorsal, dentate gyrus three weeks following the last intranasal dose (Fig. 3A; Table S2; $F(1,230) = 6.61$, $p = 0.011$, $\omega^2_{partial} = 0.10$). Males had higher densities of DCX+ cells overall ($F(1,60) = 16.70$, $p = 0.0001$, $\omega^2_{partial} = 0.23$), but there was no interaction of sex and treatment ($F(1,60) = 1.98$, $p = 0.165$, $\omega^2_{partial} = 0.08$).

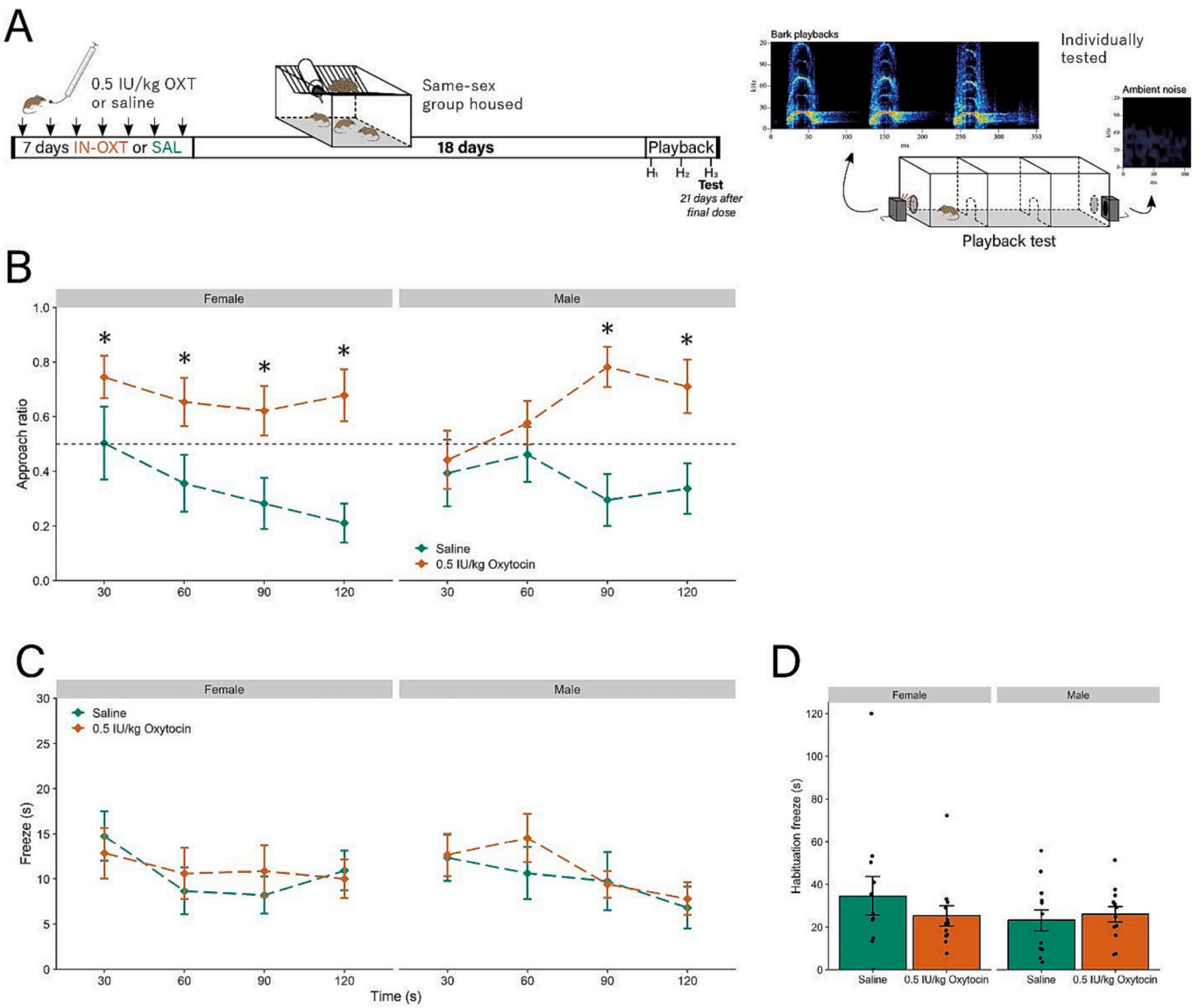

Fig. 2. Chronic intranasal oxytocin (IN-OXT) altered behavior following a long delay. **A**, Timeline for chronic IN-OXT treatment, delay, and acoustic eavesdropping test. Spectrograms represent the audio playbacks used for the tests, where one speaker played bark playbacks while the other played ambient room noise. **B**, Approach to the playbacks significantly increases in both females and males given IN-OXT. **C**, Freezing during the task is not affected by treatment, although all groups significantly decrease their freezing over the course of the test. **D**, Freezing prior to the test, during a time when no social information is present, is unchanged by treatment. Bars indicate ± SEM. *$p < 0.05$.

3.3. Ventral hippocampal neurogenesis is negatively correlated with approach ratio and freezing after a 3-week delay

Immature neurons in the ventral hippocampus may impact social behavior (Opendak et al., 2016). A linear regression predicting average approach ratio from average ventral doublecortin density for each mouse in the 3-week delay experiment revealed a significant negative correlation, with no effect of chronic IN-OXT or sex on the correlation (Fig. 3D; Table S3; $F(1,40) = 4.40$, $p = 0.042$, $R^2 = 0.41$). Follow-up analyses demonstrated that time in the approach chamber was negatively correlated with immature neuron density (Fig. S2A; $F(1,40) = 5.35$, $p = 0.026$, $R^2 = 0.39$, while time in the avoidance chamber was positively correlated with density (Fig. S2B; $F(1,40) = 4.12$, $p = 0.049$, $R^2 = 0.40$). A separate linear regression revealed that ventral neurogenesis is negatively correlated with freezing, with no effect of IN-OXT or sex on the correlation (Fig. 3E; Table S4; $F(1,40) = 6.43$, $p = 0.015$, $R^2 = 0.22$).

3.4. Chronic intranasal oxytocin did not alter behavior after a 3-day delay

Chronic OXT can increase anxiety-like behaviors during or shortly after the cessation of treatment in mice and rats (Peters et al., 2014; Winter et al., 2021). Such changes may be related to the differences in neurogenesis observed in our long delay vs short delay groups. In order to determine if the short-term effects of chronic IN-OXT differ from the increase in approach towards the playback stimulus observed three weeks following the cessation of treatment, we performed the playback test three days following treatment. Chronic IN-OXT did not affect the discrimination ratio for females or males (Fig. 4B; Table S1; $F(1,44) = 0.009$, $p = 0.925$, $\omega^2_{partial} < 0.01$). There was also no relationship between time and treatment ($F(1,44) < 0.001$, $p = 0.814$, $\omega^2_{partial} = -0.01$). Due to lack of significance of the full model, post-hoc analysis of the significance at each time point was not assessed.

Chronic IN-OXT did not affect freezing during the approach test in females or males (Fig. 4C; Table S1; $F(1,44) = 0.69$, $p = 0.410$, $\omega^2_{partial} =$

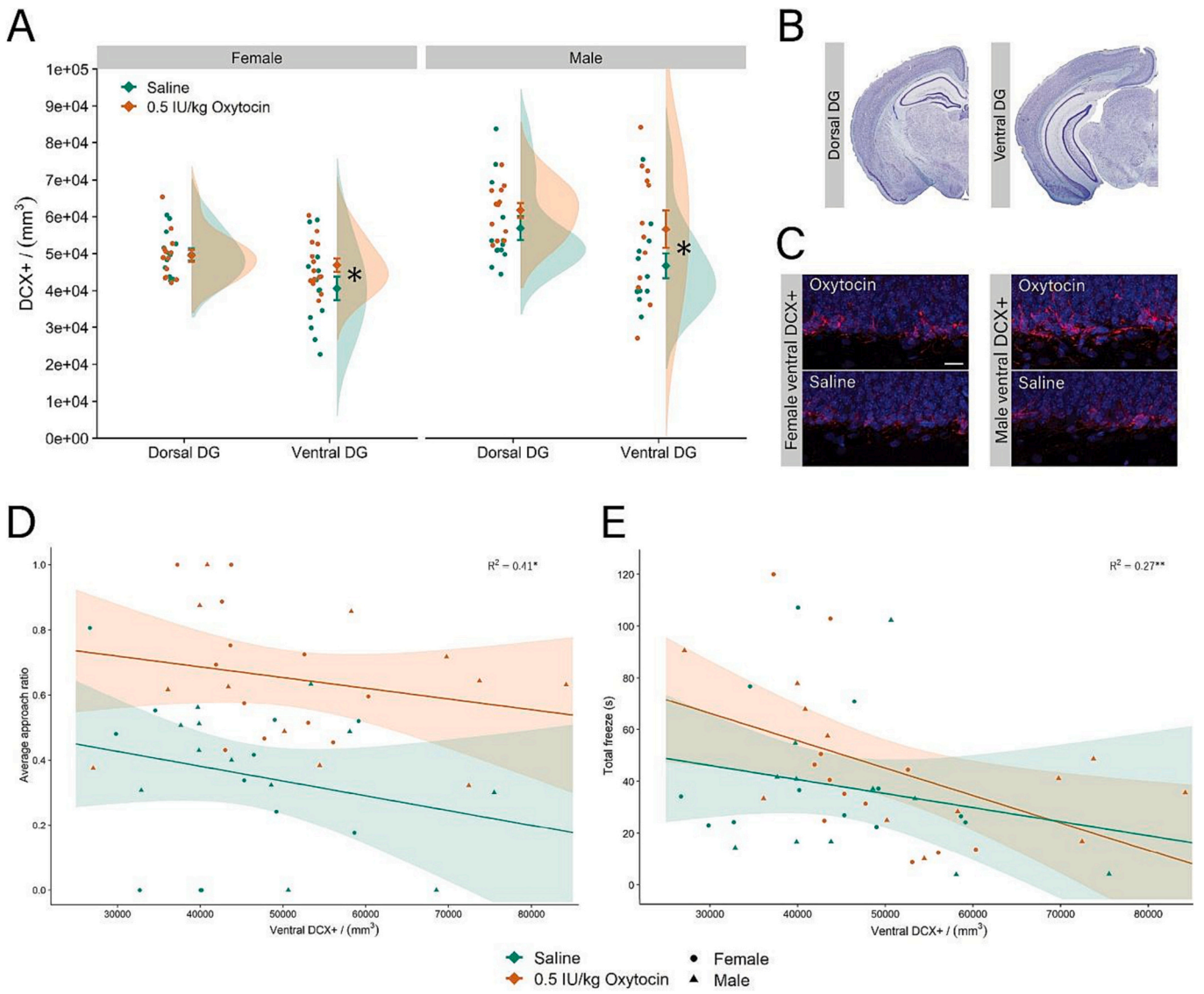

Fig. 3. Chronic intranasal oxytocin (IN-OXT) increased neurogenesis following a long delay. **A**, Example nissl stains of dorsal and ventral California mouse dentate gyrus (from the California mouse brain atlas https://brainmaps.org/index.php?p=show-california-mouse). **B**, Chronic IN-OXT significantly increases female and male ventral dentate gyrus DCX density 3 weeks after treatment cessation. **C**, representative confocal images of DCX staining. Scale bar, 20 μm. **D**, Ventral DCX density significantly negatively correlates with average approach ratio during the test for females and males. **E**, Ventral DCX density significantly negatively correlates with total freezing during the test for females and males. Bars indicate ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

0.10). As in the long delay experiment, both females and males habituated to the stimulus over time as reflected by decreased freezing ($F(1,140) = 31.32$, $p < 0.0001$, $\omega^2_{partial} = -0.26$). Chronic IN-OXT did not affect vigilance during the playback test in females or males (Fig. S3; $F(1,44) = 2.16$, $p = 0.15$, $\omega^2_{partial} = 0.16$), although there was a main effect of time whereby individuals exhibited less vigilance by the end of the test ($F(1,140) = 16.78$, $p < 0.0001$, $\omega^2_{partial} = -0.21$). Chronic IN-OXT also did not alter freezing in females or males during the pre-test habituation period (Fig. 4D; $F(1,44) = 0.30$, $p = 0.586$, $R^2 = 0.03$).

3.5. Chronic intranasal oxytocin did not increase adult neurogenesis after a 3-day delay

While it takes several weeks in other rodent species for neuroblasts in the adult hippocampus to develop into immature neurons (Yagi et al., 2020), the maturation of new-born cells in adult California mouse hippocampus has not been well-characterized and we could not rule out the possibility of a faster maturation timeline. In order to determine whether exogenous OXT rapidly induces neuronal development, we investigated the effects of chronic OXT treatment on DCX+ cell density three days following the last intranasal dose in a separate cohort of mice. Chronic IN-OXT did not alter immature neuron density in either females or males (Fig. 5A; Table S2; $F(1,52) = 0.05$, $p = 0.818$, $\omega^2_{partial} = 0.02$).

3.6. Ventral hippocampal neurogenesis is not correlated with approach ratio or freezing after a 3-day delay

Because ventral neurogenesis correlated with approach ratio and freezing following a 3-week delay, we investigated the same correlations following a 3-day delay. A linear regression predicting average approach ratio from average ventral doublecortin density revealed no evidence of a relationship (Fig. 5B; Table S5; $F(1,37) = 1.76$, $p = 0.19$, $R^2 = 0.11$). Similarly, a separate linear regression revealed no evidence that ventral neurogenesis is correlated with freezing (Fig. 5C; Table S6; $F(1,37) = 1.43$, $p = 0.240$, $R^2 = 0.08$).

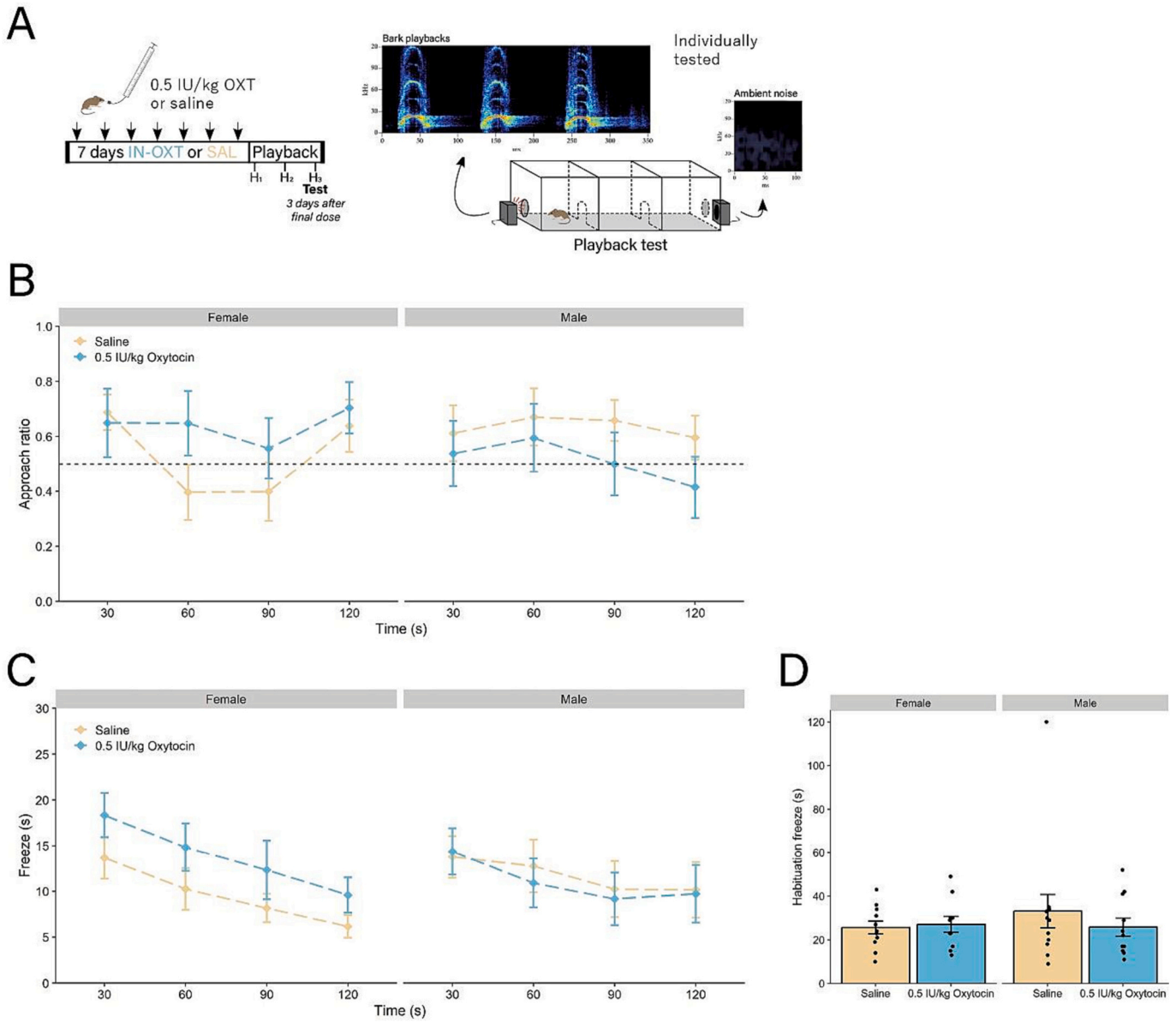

Fig. 4. Chronic intranasal oxytocin (IN-OXT) did not affect behavior following a short delay. **A**, Timeline for chronic IN-OXT treatment, delay, and acoustic eavesdropping test. Spectrograms represent the audio playbacks used for the tests, where one speaker played bark playbacks while the other played ambient room noise. **B**, chronic IN-OXT does not affect approach to playbacks following a short delay. **C**, Freezing during the task is not affected by treatment, although all groups significantly decrease their freezing over the course of the test. **D**, Freezing prior to the test, during a time when no social information is present, is unchanged by treatment.

3.7. Change in approach strategy throughout the test predicts the ratio of ventral to dorsal neurogenesis

Complex behavioral decision-making, such as the choice not to investigate a social conspecific, likely relies on cortical and subcortical processes. The dorsal and ventral hippocampus project to different areas, and neurogenesis in either region may result in different behavioral outcomes (Kheirbek and Hen, 2011). In an exploratory analysis, we correlated ventral neurogenesis and dorsal neurogenesis for all mice with quadratic change in approach ratio from the beginning 30s of the test to the final 30s of the test and observed a significant interaction between region and change in approach ratio (Fig. S4A; $F(1,178) = 5.93$; $p = 0.017$, $R^2 = 0.14$). Moreover, there was a significant quadratic relationship between change in approach ratio and the ratio of ventral to dorsal neurogenesis (Fig. S4B; $F(1,87) = 5.77$; $p = 0.019$, $R^2 = 0.14$).

4. Discussion

Oxytocin (OXT) plays a significant role in modulating various aspects of social behavior. However, the time scale at which OXT exerts its effects and its influence on behaviors related to social information gathering remain poorly understood. Our findings reveal that chronic intranasal OXT (IN-OXT) has enduring effects on approach/acoustic eavesdropping in the California mouse, a species known for its monogamous and territorial nature, particularly in relation to vocal signals. Notably, individual female and male mice exhibited increased approach behavior towards the playbacks of aggressive vocalizations between two unseen conspecifics if they had completed a chronic IN-OXT series 3 weeks prior, but not 3 days prior. Our previous research found that individual female mice did not produce calls in response to vocalization playbacks, suggesting that the observed approach behavior in the current study is a facet of social information gathering. Additionally, acute

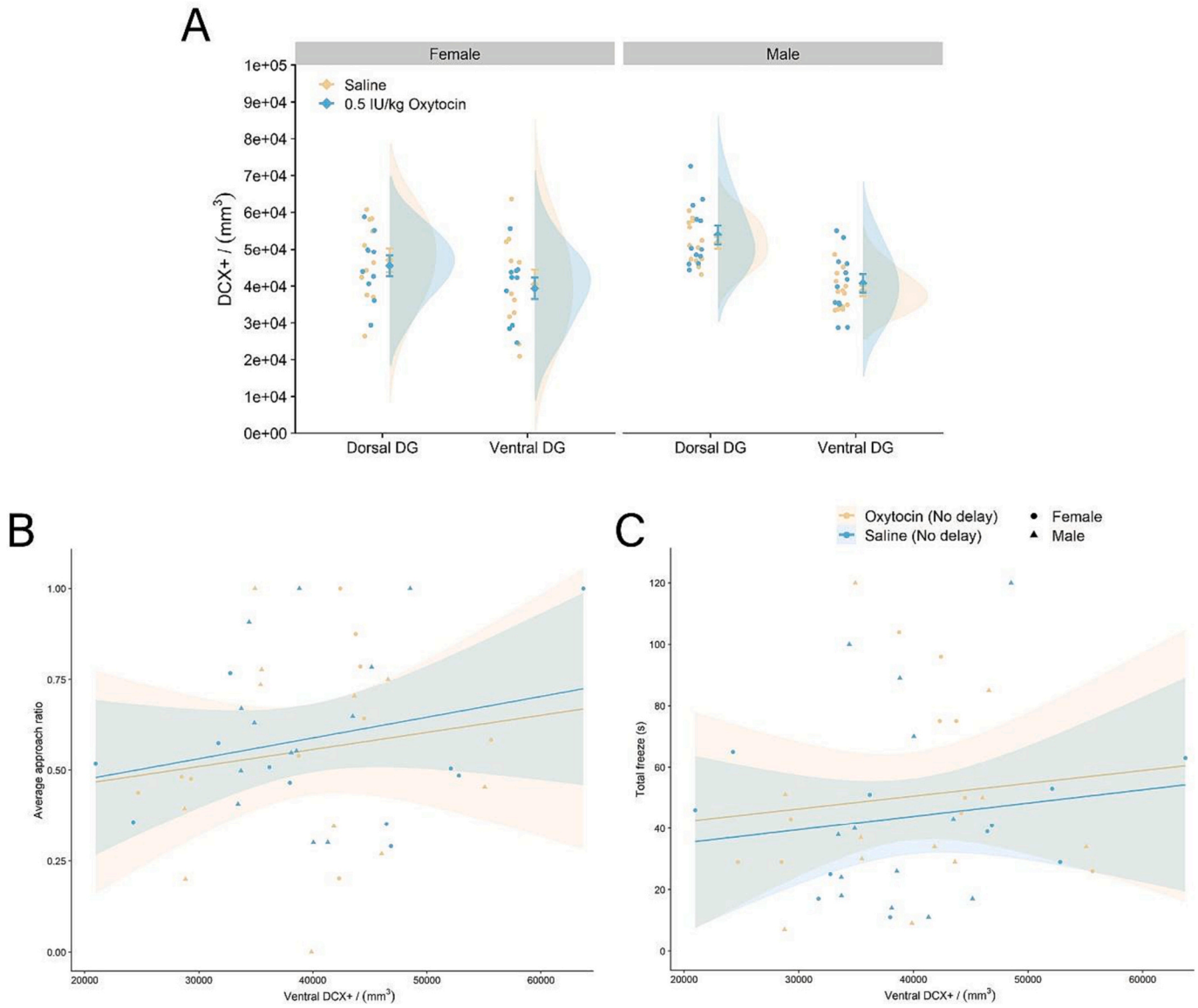

Fig. 5. Chronic intranasal oxytocin (IN-OXT) did not affect neurogenesis following a short delay. **A**, Chronic IN-OXT does not change female and male dorsal or ventral dentate gyrus DCX density 3 weeks after treatment cessation. **B**, Ventral DCX density did not correlate with average approach ratio during the test for females and males. **C**, Ventral DCX density did not correlate with total freezing during the test for females and males. Bars indicate ± SEM.

OXT administration did not result in short-term changes in approach behavior, emphasizing the significance of long-term exposure to OXT in eliciting effects in individuals in the absence of other social stimuli.

We conducted further investigations to explore whether neurogenesis serves as a mechanism underlying the observed long-term behavioral changes induced by chronic OXT treatment. Intriguingly, we found no significant behavioral change 3 days after the termination of chronic treatment, in contrast to the significant changes observed at the 3-week post-treatment time point. Concomitant with this behavioral change at the 3-week time point, neurogenesis increased in response to the chronic OXT treatment. Thus, we expected to observe a positive relationship between neurogenesis and approach/acoustic eavesdropping, even when controlling for the neurogenic effects of OXT. However, our findings revealed an unexpected negative association. Therefore, it is unlikely that the neurogenesis induced by OXT can fully explain the OXT-induced approach behavior, suggesting the involvement of other long-term mechanisms. This raises the question of the functional role of neurogenesis in the ventral hippocampus broadly, as well as the question of the functional role of OXT-induced neurogenesis. Our results align with previous well-designed studies conducted in other rodents (Anacker et al., 2018; Hill et al., 2015), highlighting an important link between anxiety and neurogenesis in the ventral hippocampus. We observed a negative correlation between neurogenesis and both freezing behavior and approach, thereby supporting the classical findings associating neurogenesis and anxiety.

Taken together, these results suggest the presence of time-dependent long-term plasticity mechanisms influenced by chronic IN-OXT as well as a role for both OXT signaling and adult neurogenesis in social information gathering strategies. Our study design is important for several reasons: 1) it systematically investigates social approach at two plasticity timepoints (short delay and long delay) that are beyond the clearance of exogenous OXT from the body (Leng and Sabatier, 2016); 2) it uses IN-OXT administration, mirroring intranasal delivery in human preclinical trials (Quintana et al., 2021); 3) it examines social approach from the perspective of vocal communication while controlling for other social cues; 4) it demonstrates the effect of chronic OXT signaling on social approach to vocalizations indicative of an unseen aggressive encounter; and 5) it enables the correlation of immature neuron density

with behavior. Below we expand on our findings and place them in context.

4.1. Timeline of OXT effects on behavior

OXT exerts complex effects on social behavior, capable of inducing both social approach and social avoidance, depending on the internal state and social context of the animal. Our results from the current study and previous study are beginning to reveal a complex effect of OXT contingent upon temporal dynamics. Here we examine single individuals of both sexes and find a lasting effect of chronic IN-OXT at 3 weeks but not at 3 days after cessation of treatment, specifically in relation to approach behavior towards playbacks of conspecific aggression calls. In a previous study we found that 5 min after a single dose of IN-OXT also had no effect for individual female approach to the playbacks (Monari et al., 2021). However, social conditions significantly alter this response. For instance, when mated females were administered a single dose of IN-OXT they exhibited an increased joint approach with their male partner 5 mins after the dose. These findings suggest the existence of OXT-sensitive mechanisms and brain regions that mediate rapid versus long-term responses, which may undergo adjustments due to the formation of affiliative pair bonds.

4.2. OXT and approach behavior

In examining the functions of OXT on approach behavior we can consider the context and underlying motivations of the observed responses. In our previous studies, we interpreted the approach behavior of a pair of mice towards an intruder or a playback of aggressive calls as a defensive response aimed at protecting either their own territory, characterized by vocalizations and aggressive behaviors, or their pair bond (Rieger et al., 2019, 2021, 2022). In the current study, we specifically investigate the effect of chronic OXT on the approach behavior of a single individual in a neutral arena and not in a chamber in which they had residency, representing a territory (Bester-Meredith et al., 2005). We propose that in this context, OXT is influencing acoustic eavesdropping behavior, a form of social information gathering (Bonnie and Earley, 2007; Liu and Lai, 2021). As we have previously demonstrated with this paradigm, both female and male mice will approach the playback of aggressive barks without vocalizing or displaying aggressive behaviors (Monari et al., 2021). Our observations lend support to the notion that a single individual, when removed from its natal group, expresses eavesdropping behavior and refrains from aggressive engagement with the playbacks. It is important to note that the focal mice in our study were not residents, were previously housed with same-sex conspecifics, and then were placed in an unfamiliar testing apparatus with no territorial or resource-related factors to defend. Moreover, they were exposed to a low-stress/challenge situation involving two fighting animals, with no sensory stimuli other than the acoustic calls. While the decision of a focal animal to engage with novel individuals and the manner in which it engages (prosocial or aggressive) are highly context-dependent, we propose that social information gathering serves as a critical initial step in all cases.

4.3. Neurogenesis and acoustic eavesdropping

The proposed link between neurogenesis and the learning and production of communicative signals in songbirds (Brenowitz and Larson, 2015; Rose et al., 2022) underscores the potential significance of neurogenic processes in communication. However, the connection between neurogenesis and the perception and attunement to communicative signals remains relatively unexplored, particularly in mammals. Adult neurogenesis is linked to anxiety regulation and social recognition (Cope et al., 2020; Hill et al., 2015), likely through its ability to suppress downstream hippocampal activity (Anacker et al., 2018). Both anxiety and social recognition mediate social approach (Duque-Wilckens et al., 2018; Lagace et al., 2010) and are therefore critical for social information gathering. In our study, we observed a negative correlation between ventral neurogenesis and freezing behavior, supporting previous research linking increased neurogenesis to enhanced resilience against anxiety-like responses. For example, (Anacker et al., 2018) demonstrated that inhibiting adult neurogenesis in the ventral hippocampus increased susceptibility to social defeat stress, whereas enhancing neurogenesis promoted resilience to chronic stress. Additionally, we found a surprising negative correlation between ventral neurogenesis and approach behavior to the playbacks of aggressive conspecific vocalizations, suggesting a potential relationship between neurogenesis and social information gathering strategies. Our exploratory analysis showing that intra-task changes in approach or avoidance strategies are linked with the ratio of ventral to dorsal neurogenesis may reflect the computational complexities of this decision-making process, as proportionally higher ventral or dorsal neurogenesis may represent greater influence of downstream limbic or cortical activity, respectfully (Fanselow and Dong, 2010). Further characterization of the observed changes in neurogenesis may help illuminate the role of OXT on neurogenesis, and neurogenesis in social decision making in future studies. For example, doublecortin-positive neurons can be categorized based on developmental stage as proliferative, intermediate, or post-mitotic (Plümpe et al., 2006; Workman et al., 2015). DCX+ cells are, however, young neurons that are on average less than one month old (Mahmoud et al., 2016), thus the sum of DCX+ neurons can be considered as a measure of neurogenesis even if it does not allow a precise determination of timing. Taken together, these findings illuminated the complex interplay between neurogenesis, social behavior, and communication, underscoring the need for further investigation in this area.

5. Conclusion

Our study provides evidence that chronic IN-OXT exerts enduring effects on acoustic eavesdropping and structural plasticity. Furthermore, our findings are the first to indicate that ventral adult neurogenesis plays a role in social information gathering. These results lend support to the hypothesis that OXT modifies complex social decision making, at least partially, through the promotion of neurogenesis in the ventral hippocampus.

Data availability

Data available from the Open Science Framework: https://osf.io/dcef6/.

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Research was conducted at the University of Wisconsin-Madison, which occupies the ancestral Ho-Chunk land known as Teejop. Following an 1832 treaty, federal and state governments repeatedly, but unsuccessfully, sought to forcibly remove the Ho-Chunk from Wisconsin (Loew, 2013). As members of a land grant institution we directly benefit from land theft, and we challenge ourselves and others to reflect on the perpetuation of the colonialist roots of western scientific progress. Z. Herro and J. Bymers conducted data collection and manuscript editing. A. Auger, L. Riters, F. Madison, E. Hammond, and C. Malone provided manuscript feedback. We also thank the UW-Madison animal research technicians. Microscopy was performed at the Newcomb Imaging Center, Department of Botany, University of Wisconsin – Madison. Research was supported by the National Science Foundation (IOS-1946613 and DGE-1747503).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2023.105443.

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