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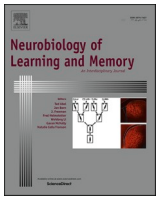

Sexually dimorphic muscarinic acetylcholine receptor modulation of contextual fear learning in the dentate gyrus

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ABSTRACT

Contextual fear conditioning, where the prevailing situational cues become associated with an aversive unconditional stimulus such as electric shock, is sexually dimorphic. Males typically show higher levels of fear than females. There are two components to contextual fear conditioning. First the multiple cues that encompass the context must be integrated into a coherent representation, a process that requires the hippocampus. The second is that representation must be communicated to the basolateral amygdala where it can be associated with shock. If there is inadequate time for forming the representation prior to shock poor conditioning results and this is called the immediate shock deficit. One can isolate the contextual processing component, as well as alleviate the deficit, by providing an opportunity to explore the context without shock prior to the conditioning session. The purpose of the present study was to determine the extent to which cholinergic processes within the dentate gyrus of the hippocampus during contextual processing contribute to the sexual dimorphism. Clozapine-n-oxide (CNO) is a putatively inactive compound that acts only upon synthetic genetically engineered receptors. However, we found that CNO infused into the dentate gyrus prior to exploration eliminated the sexual dimorphism by selectively decreasing freezing in males to the level of females. Biological activity of CNO is usually attributed to metabolism of CNO to clozapine and we found that clozapine, and the muscarinic cholinergic antagonist, scopolamine, produced results similar to CNO, preferentially affecting males. On the other hand, the muscarinic agonist oxotremorine selectively impaired conditioning in females. Overall, the current experiments reveal significant off-target effects of CNO and implicate muscarinic cholinergic receptors in the dentate gyrus as a significant mediator of the sexual dimorphism in contextual fear conditioning.

Contextual fear conditioning occurs when an aversive event becomes associated with the prevailing situational cues present at the time the aversive stimulus is presented (Fanselow, 1980). Thus, the context acts as a conditional stimulus (CS) that becomes associated with the aversive unconditional stimulus (US) that occurred in that situation. In typical laboratory experiments brief footshock usually serves as the aversive US. Contextual fear conditioning is sexually dimorphic with males showing higher levels of conditioning than females, especially with weak training parameters (e.g., 1 trial conditioning, Maren, De Oca, et al., 1994; Wiltgen et al., 2001).

In traditional conditioning with a unimodal temporally discrete CS, conditioning decreases as time between CS onset and US onset increases. This CS-US relationship is reversed in contextual fear conditioning, which increases as the time between entry into the context and delivery of the US is lengthened (Fanselow, 1986, 2010; Zinn et al., 2020). This period between placement in the context and shock delivery is called the placement-to-shock interval (PSI). The deficit in conditioning with very short PSIs (e.g., 30 sec or less) is called the immediate shock deficit (Fanselow, 1986). The immediate shock deficit is more pronounced in females and the sex difference disappears with long PSIs (e.g., 720 s, Wiltgen et al., 2001). Conceptual accounts of the immediate shock deficit suggest that this preshock time is needed to integrate the myriad of features that comprise the context into a unified representation before the context can be used effectively as a CS (Fanselow, 2000; Krasne et al., 2015). Support for this idea comes from the finding that providing a period for exploration that is discontiguous with the US reduces the

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amount of preUS exploration needed for learning to occur (Fanselow, 1990). That is, safe pre-exposure to the context prior to conditioning alleviates the immediate shock deficit. Interestingly, pre-exposure to the context also reduces the difference in conditioning between males and females (Wiltgen et al., 2001).

If the shock US is signaled by a discrete CS, the shock will support fear conditioning to both the discrete and contextual CS. Since hippocampal disruption impairs conditioning to the contextual CS, but not the discrete CS, we have suggested that the hippocampus is critical for the integration process that occurs during exploration as opposed to the formation of the CS-US association (Fanselow, 2000; Kim & Fanselow, 1992). This binding of contextual attributes depends on extensive interactions between the hippocampus and cortex (Bucci & Robinson, 2014; Krasne et al., 2015; Yavas et al., 2019). Consistent with the hypothesis that the hippocampus is selectively involved in context processing as opposed to Context-US association formation, manipulations of hippocampal function only during context pre-exposure will alter the benefits of pre-exposure (Matus-Amat et al., 2004). For example, blockade of hippocampal n-methyl-D-aspartate (NMDA) glutamate receptors, or muscarinic cholinergic receptors, only during pre-exposure eliminates the contextual pre-exposure facilitation effect (Pinizzotto et al., 2020; Stote & Fanselow, 2004). Besides these loss of function experiments, optogenetic enhancement of hippocampal cholinergic activity during pre-exposure further facilitates later conditioning at short PSIs (Hersman et al., 2017).

While the entire hippocampus participates in contextual fear conditioning, here we focus on dentate gyrus. Deletion of NMDA receptors from the dentate impair rapid contextual fear discrimination (McHugh et al., 2007). Activating dentate neurons that were active during contextual fear memory will trigger a fear response in a safe context (Liu et al., 2012). Contextual fear conditioning correlates with long-term potentiation (LTP) of dentate granule cells produced by perforant path stimulation and manipulations that enhance dentate LTP also enhance context conditioning (Maren, De Oca, et al., 1994, Maren, DeCola, et al., 1994). Interestingly, besides showing heightened contextual fear, male rats exhibit greater dentate LTP than females (Maren, De Oca, et al., 1994). Furthermore, muscarinic cholinergic activation enhances dentate LTP (Burgard & Sarvey, 1990; Frey et al., 2003).

As mentioned above, the sexual dimorphism is most apparent with weak conditioning parameters. For example, male rats showed enhanced contextual fear conditioning relative to females when they received one, but not three tone-shock pairings (Maren, De Oca, et al., 1994). Males and females were not different in their responses to the trained auditory cue. That the dimorphism was specific to contexts suggests that males and females process contexts differently. Since increasing the number of trials eliminated the dimorphism, it seems to be that the rate, not the asymptote, of learning differs between sexes. CS salience is the major determinant of the rate of conditioning (Rescorla & Wagner, 1972). Raza et al. (2017) suggest that muscarinic receptors in the dentate gyrus regulate contextual salience; however, Raza et al. only examined male mice. Therefore, we decided to investigate the extent to which dentate gyrus cholinergic activity contributed to the sexual dimorphism in contextual fear conditioning.

To isolate contextual processing, we chose to manipulate cholinergic activity only during context pre-exposure. Initially, our intent was to use chemogenetic manipulation of cholinergic activity in the dentate gyrus of ChAT::Cre rats. Chemogenetic studies employ mutated muscarinic receptors that are insensitive to acetylcholine but do respond to the artificial ligand clozapine-*N*-oxide (CNO), an analog of clozapine, that is supposed to have limited action on endogenous receptors (Armbruster et al., 2007; Smith et al., 2016). These artificial receptors are called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs; Roth, 2016). However, it has been reported that CNO may have some biological activity in the absence of DREADDs, possibly because CNO undergoes systemic conversion to clozapine (Gomez et al., 2017; MacLaren et al., 2016; Manvich et al., 2018). Therefore, we started our work with pilot studies to make sure that dentate infusion of CNO was inactive in rats that did not have DREADDs. Surprisingly, we discovered that dentate infusion of CNO alone was capable of eliminating the difference between males and females. To determine the source of the difference we first tested if clozapine would produce a similar result, which it did. Clozapine's biological action is complex but some of its effects are mediated by muscarinic cholinergic receptors (Baldessarini & Frankenburg, 1991; Ereshefsky et al., 1989; Jann, 1991; Jann et al., 1993; Shirazi-Southall et al., 2002) and as pointed out above, acetylcholine is a key regulator of context processing. Therefore, we further investigated the action of a cholinergic agonist (oxotremorine) and antagonist (scopolamine) when infused directly into the dentate on the sexual dimorphism in contextual fear conditioning. The results suggest that muscarinic cholinergic receptors play a major role in the sexual dimorphism of contextual fear conditioning.

1. Materials and methods

1.1. Subjects

Male (300–350 g) and female (250–300 g) Chat::Cre rats of Long-Evans background (n = 140, see figure legends for individual group sizes) were used for all experiments. The breeding stock for these rats was originally provided by Witten and Deisseroth but were bred in our in-house breeding colony for multiple generations (Witten et al., 2011). Animals were pair-housed prior to surgery and singly housed following surgery. The colony was maintained at 22 °C (±1°C) with a 12-hour light/dark cycle. The rats had *ad libitum* access to regular rat chow and water. Animals were handled for 7 to 10 days prior to the start of the experiments, which were carried out in accordance with policy set and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

1.2. Surgery

After handling, animals underwent stereotaxic surgery and guide cannula (Plastic One, VA, USA) were implanted bilaterally. Following anesthetization with a mixture of oxygen and isoflurane (1–5%), veterinary ophthalmic ointment was applied to the eyes to protect them from drying and debris. The heads were shaved, and the surface of the skull was exposed and cleaned. After the holes were drilled by hand, guide cannulae (4.5 mm below pedestal; 26 GA 38172) were then lowered to target the dentate gyrus (3.5 mm posterior to bregma; 2.3 mm lateral to the bregma). Early placement check surgeries revealed a sex difference in the ideal depth to target the dentate gyrus. Adjusting for this, the DV coordinates were subsequently set to 3.0 mm for males and 2.5 mm for females. Guide cannulae were secured with stainless steel anchor screws (Plastic One), and to aid attachment, dental acrylic was used to fix the cannula to the skulls. Dummy cannulae (4.5 mm C315G) were inserted into the guide cannulae and replaced regularly prior to and following drug infusion. Physiological saline (5 ml, s.c) was injected to prevent dehydration, and an analgesic (carprofen, 5 mg/kg, s.c) was administered before and 24 h after surgery. Pre- and post-operative treatments were carried out in accordance with the guidance of the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

1.3. Chemicals and drugs

Clozapine *N*-oxide (CNO) dihydrochloride (water soluble) and clozapine dihydrochloride (water soluble) were purchased from Hello Bio Inc. (Princeton, NJ, USA). Scopolamine hydrobromide, Oxotremorine M, and aCSF were purchased from Tocris (Minneapolis, MN, USA). Stock solutions of drugs were made and working concentrations of the drugs were prepared freshly on the day of each experiment by appropriate dilutions of the stock solutions in aCSF.

1.4. Apparatus

Behavioral experiments were conducted in Med Associates fear conditioning chambers (VFC-008; 30 × 25 × 25 cm), and all procedures were programmed and controlled by VideoFreeze software (Med Associates, Inc., St. Albans,VT). The grid floor consisted of 16 stainless steel rods (3/8″) spaced 1.6 cm apart. The fear conditioning chamber was kept at room temperature and was equipped with fans (60 dB) above the chamber that served as background noise. The light within the fear conditioning chamber and the experimental room light were both left on during the experiment. The chamber was cleaned and scented with a 70% isopropyl alcohol solution between each session and animal. Animals were transported to the laboratory area using a portable cart covered with a black sheet and were then placed in a separate room that did not contain any cues associated with the surgery and/or training rooms prior to behavior and/or drug infusion.

1.5. Procedure

Each experiment employed a three-day conditioning protocol (Fig. 1). On Day 1, following transport to the lab, all rats received bilateral infusions of drug or vehicle into the dentate gyrus of the hippocampus prior to placement in the fear conditioning chambers. All rats were infused with 300 nl per hemisphere, one hemisphere at a time, at a rate of 150 nl/min. These drugs were delivered via infusion pump (Harvard Apparatus, South Natick, MA, United States), to which the implanted cannulae were connected to PE-20 polyethylene tubing (Plastic One) connected to a 10 ul Hamilton syringe. Rats were held by experimenters throughout drug delivery. After the infusions, the injection cannulae were left in place for a few minutes to make sure delivery was complete and prevent backflow of the drug. Dummy cannulae were then reinserted, and animals were placed in the fear conditioning chambers.

On Day 1, following drug infusion, the animals received 10 min of exposure to the context without receiving any footshock/US. During this pre-exposure to the to-be-shocked context, we observed no drug-induced behavioral changes and freezing was near 0 in all animals. On Day 2, the rats were returned to the same context, and received a single footshock (1 mA, 2 s) after a 30-second placement-to-shock interval (PSI). They were removed from the context after a further 30 s. On Day 3, the rats were again returned to the same context for a 10-minute contextual fear memory test. Freezing throughout all three days was analyzed using VideoFreeze software and scored as periods of time in which the animal's motion was below a threshold calibrated to mimic scores determined by experienced observers.

Rats did not receive a drug infusion on the day of shock or testing; rather, animals were only given infusions prior to pre-exposure to the context, a crucial period in which animals learn about the context in a hippocampus-dependent manner. For each experiment, one group of animals was infused with an active drug and one group was infused with the same volume of aCSF. In Experiment 1 rats were either infused with: aCSF or CNO (1 mM). The concentration for CNO was chosen based on chemogenetic studies using intracranial injection of CNO to activate DREADDs (Ge et al., 2017; Mahler et al., 2014; McGlinchey & Aston-Jones, 2018; Shipman et al., 2019; Venniro et al., 2017). Experiment 2 had the same design except clozapine (1 mM) was substituted for CNO. Experiment 3 administered scopolamine hydrobromide (50 mg/ml). This concentration for scopolamine was chosen in accordance with previous contextual fear experiments (Gale et al., 2001; Hersman et al., 2019). Experiment 3 used Oxotremorine-M (300 ng/ml) whose concentration was chosen based on a previous study that characterized its effects on memory consolidation (Sánchez-Resendis et al., 2012).

1.6. Histology

Following the behavioral experiments, the rats were anesthetized with isoflurane and sacrificed via cervical dislocation for further histological analyses to assess cannulae placements. The rats' brains were removed from their skulls and placed in a 4% PFA solution overnight, then transferred to a 30% sucrose solution in PBS for 3 days until they sank. Coronal sections of 40 μm thickness that contained the hippocampus were sliced using a cryostat. Injection sites were reconstructed using a bright field microscope (Keyence BZ-X710 fluorescent microscope), and rats with cannulae outside the target structure were excluded from any behavioral analysis (Fig. 2).

1.7. Data analysis

Data were collected using VideoFreeze software to automatically score freezing behavior. Briefly, all data were analyzed using the general linear model in SPSS. For each experiment, omnibus multifactorial ANOVA were initially performed. Higher order and simple interactions were followed up with post-hoc analyses using Bonferroni-corrected comparisons. For repeated measures ANOVA, the Huynh-Feldt correction was used when sphericity was violated. While unadjusted degrees of freedom are presented in order to help identify group sizes, p values reflect corrections for sphericity.

2. Results

2.1. CNO and clozapine impact contextual fear learning

Prior to any attempts at chemogenetic manipulation using DREADDs, rats were tested to assess whether CNO alone modulates contextual learning, in the absence of any AAV vector. CNO (1 mM) or aCSF was infused into the dentate gyrus prior to a 10-minute pre-exposure session on Day 1 in which animals could explore a fear conditioning chamber without receiving any shock. On Day 2, all rats were returned to the testing chamber and received a foot-shock (1 mA, 2 s) after a 30-second PSI. Finally, on Day 3 the subjects were returned to the

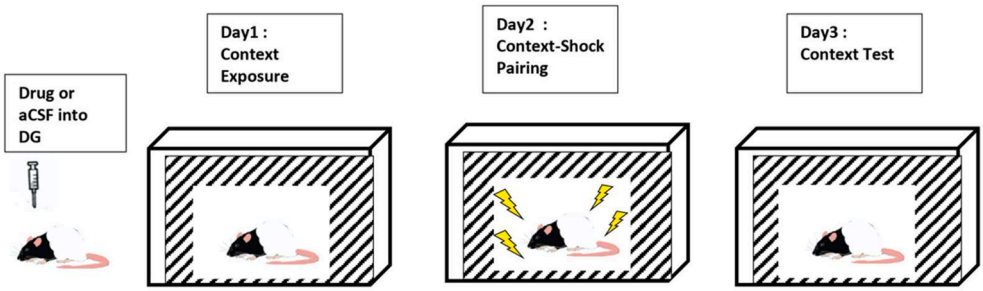

Fig. 1. Experimental design. On Day 1, the animals received drug or aCSF infusions into the dentate gyrus (DG) and were then placed into a training chamber for 10 min of context pre-exposure. On Day 2, animals were returned to the training chamber and received a foot-shock (1 mA, 2 s) after a 30-second PSI. On Day 3, animals were returned to the same context and their freezing was assessed across the 10-minute test session. Red circles are females; blue circles are males.

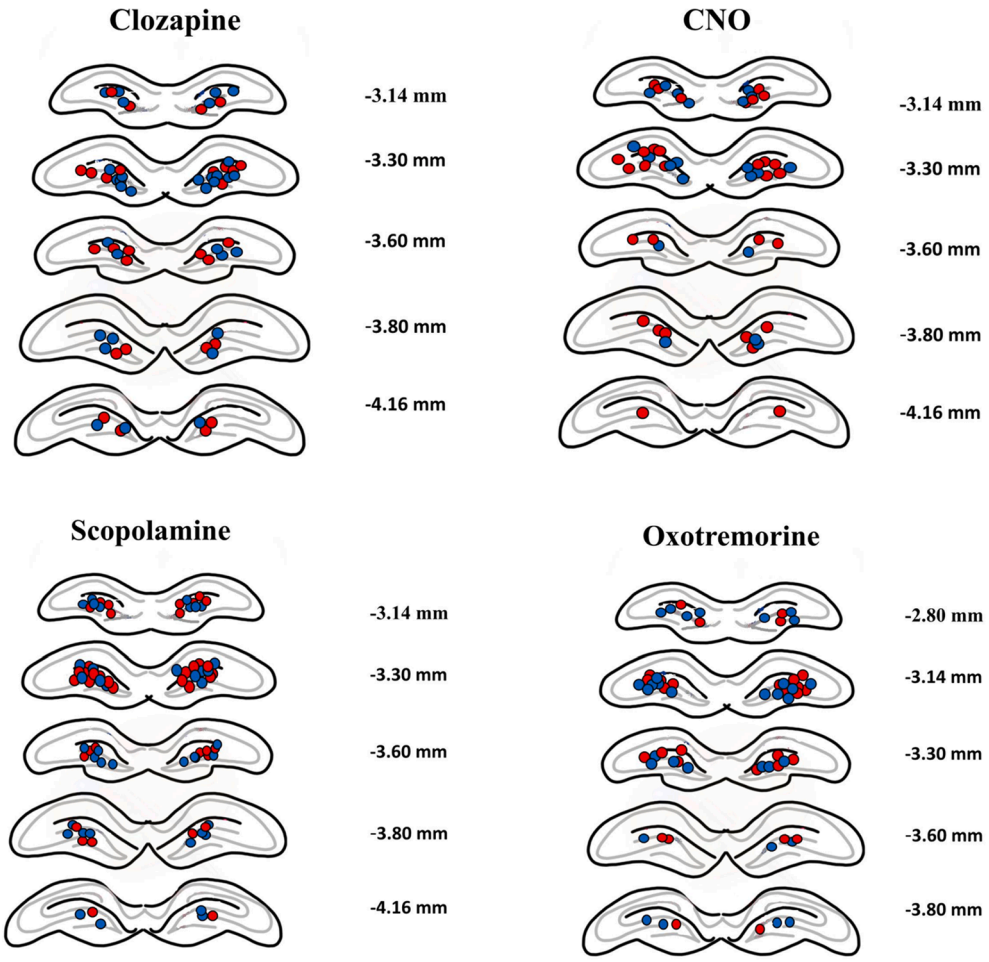

Fig. 2. Dentate Gyrus Cannulae Placement. All dentate gyrus cannulae tip placements are shown above. a) CNO; b) Clozapine; c) Scopolamine; d) Oxotremorine. Red circles are females; blue circles are males.

same context and their freezing was assessed across a 10-minute test session. Throughout pre-exposure on Day 1 and during the 30 s prior to the shock on Day 2, we observed no drug-induced behavioral changes and little freezing (<5%) was observed with no differences between groups. The data from the test session on Day 3 were analyzed for freezing behavior to assess whether CNO had an impact on contextual learning during pre-exposure (Fig. 3, left panel). The analysis revealed a significant effect of Time across the 10-minute session ($F [4,120] = 28.60$; $p < 0.0001$) because freezing was maximal at the beginning of the test. There was a significant Sex × Drug interaction ($F [1,30] = 4.51$; $p < 0.05$). As reported previously undrugged male rats froze more than females. Furthermore, CNO appeared to impair contextual learning in male but not in female rats, reducing the levels of freezing in males to that of females.

As CNO unexpectedly had effects when administered alone without any DREADD construct, we sought to determine if these effects were due to the conversion of CNO to clozapine. In the next experiment, using the same procedure, we tested whether bilateral infusions of clozapine (1

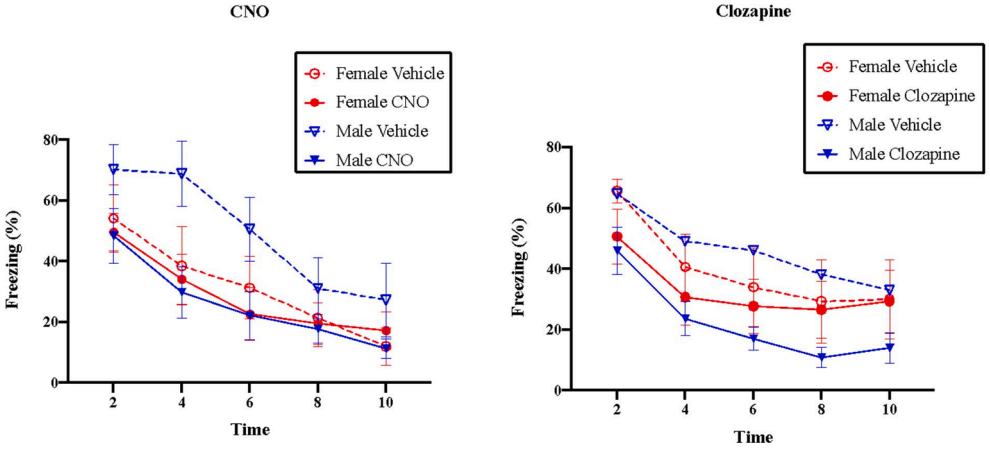

Fig. 3. CNO and Clozapine impact contextual fear. Animals are returned to the context in which they received shock and their freezing responses across minutes were assessed. For **CNO**, there was a significant **Time effect** **** $p < 0.0001$ and a significant **Sex × Drug interaction** * $p < 0.05$. Female drug ($n = 10$); female vehicle ($n = 7$), male drug ($n = 10$); male vehicle ($n = 7$). For **Clozapine**, there was a significant **Time effect** **** $p < 0.0001$ and a significant **Drug effect** * $p < 0.05$. Female drug ($n = 11$); female vehicle ($n = 8$), male drug ($n = 10$); male vehicle ($n = 7$).

mM) into the dentate gyrus would impact contextual fear learning and whether it would do so in a similar manner as CNO (Fig. 3, right panel). Analysis of freezing during the test session on Day 3 revealed significant effects of Time ($F [4,128] = 18.17$; $p < 0.0001$) and Drug ($F [1,32] = 4.51$; $p < 0.05$). While the sex difference in contextual fear within the vehicle groups was not reliable in this experiment and there was no significant interaction with sex, it appears that clozapine primarily reduced fear in males. Whether the effects of CNO and clozapine are mediated by the same mechanisms is unclear; nevertheless, infusions of both CNO and clozapine into the dentate gyrus prior to context pre-exposure and subsequent fear learning resulted in similar behavioral responses during testing.

The pharmacology of clozapine is complex and nonspecific having action on, but not limited to, several cholinergic and monoaminergic receptors (Roth & Driscol, 2011). As clozapine potently binds to muscarinic cholinergic receptors (mAChR) and these receptors have been implicated in contextual fear conditioning (Gale et al., 2001; Pinizzotto et al., 2020), we explored dentate gyrus application of a selective mAChR agonist and antagonist.

2.2. Scopolamine and oxotremorine impact contextual learning in a sexually dimorphic manner

Further analyses were carried out with oxotremorine-M, a selective muscarinic agonist, and scopolamine, a muscarinic antagonist, to test the extent to which these drugs impact contextual learning when infused during pre-exposure. The same surgical and behavioral procedures as the prior experiments were repeated. The only difference is that for one experiment, animals received either scopolamine (50 mg/ml) or aCSF prior to pre-exposure, and for the final experiment, animals received either oxotremorine (300 ng/ml) or aCSF. The data from the test session on Day 3 were again analyzed for freezing behavior to assess whether the drugs had an impact on contextual learning.

Analysis obtained from the scopolamine experiment revealed a significant effect of Time ($F [4,128] = 31.0$; $p < 0.0001$), a significant Time × Sex × Drug interaction ($F [4,128] = 2.94$; $p < 0.05$), a significant Time × Drug interaction ($F [4,128] = 3.23$; $p < 0.05$), and significant Drug × Sex interaction ($F [1,32] = 6.87$; $p < 0.05$; Fig. 4, left panel). Scopolamine, like CNO and clozapine impaired contextual fear memory in males to a higher extent than in females. Indeed, in females scopolamine tended to increase contextual memory.

Furthermore, data obtained from the oxotremorine experiment revealed a significant effect of Time ($F [4,120] = 24.24$; $p < 0.0001$), a significant Time × Sex × Drug interaction ($F [4,120] = 2.63$; $p < 0.05$), and a significant effect of Sex ($F [1,30] = 7.71$; $p = 0.009$; Fig. 4, right panel). While oxotremorine also impaired contextual fear learning in a sexually dimorphic manner, its impact was primarily on female rats, as opposed to any other drug used in these experiments. Female rats that received intracranial oxotremorine infusion prior to pre-exposure, at this particular drug concentration, showed a lower level of freezing throughout the test session than females who received vehicle infusions and male rats, regardless of drug infusion. This was not the case for the female rats that received scopolamine, as again, female rats that received scopolamine infusions behaved similar to female rats that received vehicle infusions.

These results indicate that agonizing and antagonizing mAChRs generates differential effects in each sex. The results showed that antagonizing mAChRs with scopolamine impacted contextual fear in a similar manner to CNO and clozapine, in which the drug primarily impaired contextual learning in males. However, activating mAChRs with oxotremorine did not disrupt contextual learning in males, but it did disrupt it in female rats. While we cannot explicitly determine whether the scopolamine-mediated behavior in this contextual-learning paradigm is due to the same underlying mechanisms as the CNO and clozapine-mediated responses, the results from all experiments here heavily implicate muscarinic cholinergic activity in the dentate gyrus of the hippocampus as crucial for contextual fear learning and memory.

3. Discussion

The original goal of these studies was to chemogenetically alter cholinergic activity in the dentate gyrus to determine its role in the sex differences in contextual fear conditioning that we previously reported. We could not pursue our initial strategy because our chemogenetic activator, CNO, was found to have activity independent of expression of DREADDs. However, by comparing this action of CNO to clozapine, oxotremorine, and scopolamine we obtained clear evidence that cholinergic activity is a key contributor to the sexual dimorphism in contextual fear conditioning.

Our results show that CNO and its potential back-metabolite clozapine impact contextual fear, specifically in male rats. Infusion of these drugs into the dentate gyrus of the hippocampus prior to pre-exposure to a to-be-shocked context resulted in a subsequent impairment in contextual fear memory in males. Follow-up experiments with cholinergic drugs revealed a likely role for muscarinic cholinergic receptors in the dentate gyrus in mediating both contextual fear learning generally

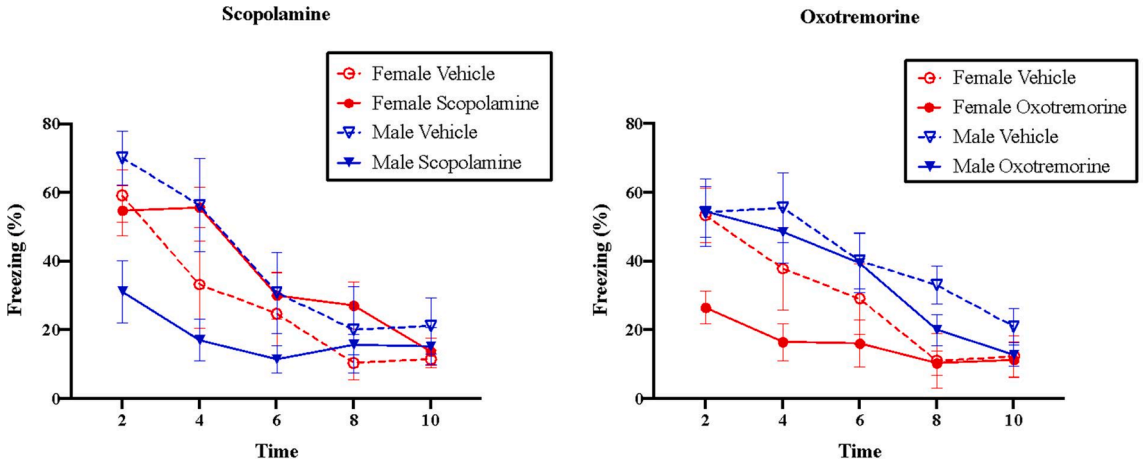

Fig. 4. Scopolamine and oxotremorine impact contextual fear in a sexually dimorphic manner. Animals are returned to the context in which they have received shock and their freezing responses across minutes were assessed. For **Scopolamine**, there was a significant effect of **Time** **** $p < 0.0001$, a significant **Time × Sex × Drug interaction** * $p < 0.05$, and a significant **Sex × Drug interaction** * $p < 0.05$. Female drug (n = 11); female vehicle (n = 8), male drug (n = 10); male vehicle (n = 7). For **Oxotremorine**, there was a significant effect of **Time** **** $p < 0.0001$, a significant **Time × Sex × Drug interaction** * $p < 0.05$, and significant effect of **Sex** *** $p = 0.009$. Female drug (n = 10); female vehicle (n = 7), male drug (n = 10); male vehicle (n = 7).

and its sexual dimorphism specifically. When infused prior to context pre-exposure, the cholinergic antagonist scopolamine resulted in a similar pattern of results as CNO and clozapine, in which males infused with the drug show impaired contextual fear memory at testing. Interestingly, infusion of the cholinergic agonist oxotremorine again impaired contextual fear, but uniquely only in female rats. These results as a whole implicate muscarinic cholinergic activity in the dentate gyrus as a potential mechanism through which contextual fear learning becomes sexually dimorphic.

In all the experiments reported here, drug infusion was conducted immediately prior to a context pre-exposure session on day 1 in which the animal was allowed to explore the context without receiving any shock. On day 2, animals received a shock after a short 30 s PSI, and on day 3 animals were tested for fear to the pre-exposed, shocked context. Thus, any impact of the drugs would have been on contextual learning processes during pre-exposure. During this pre-exposure, animals learn about features of the context and develop a contextual representation that can be retrieved on the following day prior to shock (Fanselow, 2000; Krasne et al., 2021). The short 30 s PSI on day 2 requires the rats to quickly recognize the context they were pre-exposed to in order to properly associate a shock with that context, allowing for the development of a contextual fear memory. These experiments, therefore, allowed us to evaluate how each drug impacted the development of contextual representations in both male and female rats. Cholinergic mechanisms in the hippocampus are known to participate in the formation and storage of episodic or contextual memory (e.g., Easton & Parker, 2003; Packard et al., 1996). Acetylcholine levels in the hippocampus are elevated during memory encoding and fluctuate throughout memory consolidation. Therefore, disturbing these mechanisms during contextual learning may impair the formation of contextual representations.

It should be noted that all groups in this study were pre-exposed to the context prior to conditioning. Extensive research in our laboratory has shown that with the very short period between placement in the context and shock used in these studies there is virtually no freezing without pre-exposure (Fanselow, 1990; Landeira-Fernandez et al., 2006; Stote & Fanselow, 2004; Wiltgen et al., 2001). Therefore, we omitted the nonpre-exposed condition because it would not be particularly informative with respect to the questions we posed and would create a condition of marked heterogeneity of variance that could have reduced the power of our analyses.

It has been shown that mAChRs have binding sites for clozapine. Previous studies have shown that CNO can influence behavior, perhaps directly or by reversion back to its clozapine precursor (Gomez et al., 2017; MacLaren et al., 2016; Manvich et al., 2018). The results reported here show that CNO infusions into the dentate gyrus indeed affect contextual learning and behavior as measured in a contextual fear learning procedure. Interestingly, CNO-mediated effects on contextual fear memory showed a sexual dimorphism. Female rats appear somewhat resistant to this drug-mediated memory disturbances, whereas male rats were quite strongly affected by the drug. In fact, CNO appears to be able to abolish the typical sex difference in contextual fear in which males usually show more contextual fear than females. Systemic injections of clozapine have been shown by others to impair contextual fear conditioning, but that study only tested male rats (Calzavara et al., 2009). Our data indicate that infusion of both CNO and clozapine specifically into the dentate gyrus primarily impacted male rats. While the extent to which the effects of CNO and clozapine are mediated by the same mechanisms in the dentate gyrus is not necessarily clear from these experiments, it is very likely that effects of CNO are due to back-conversion to clozapine given the similar actions of both drugs. Such back-conversion has been found in blood of both rats and mice when CNO was administered systemically (Gomez et al., 2017; MacLaren et al., 2016; Manvich et al., 2018). Our data suggests that such conversion also happens in brain. Future studies are needed to further validate that this is the case but given the small amounts of drug we administered to the dentate gyrus a highly sensitive method of detecting clozapine in brain tissue would have to be developed.

Given that both drugs can have antagonistic effects on mAChRs (Gomez et al., 2017; Li et al., 2005; Zorn et al., 1994), and that the cholinergic antagonist, scopolamine, and the agonist, oxotremorine, showed similar and opposing effects respectively, we believe CNO and clozapine exert their effect on contextual learning via local control over cholinergic mechanisms in the dentate gyrus. Indeed, muscarinic cholinergic activity in the dentate gyrus has been reported to modulate contextual fear learning (Medeiros et al., 2011; Raza et al., 2017). However, as these prior studies only utilized male subjects, a potential sexual dimorphism was not identified. Thus, the current experiments reveal a novel sex difference with regard to how mAChR activation in the dentate gyrus can modulate contextual learning.

Muscarinic antagonism and agonism differentially affected males and females. One possibility is that there is an ideal range of muscarinic activation of the dentate for maximal contextual learning and that males normally sit near the bottom of this range (see graphical abstract). If this was the case muscarinic antagonists would move the male rat below the ideal range, while agonists would still leave the animal within the ideal range. Perhaps, females normally lie slightly above this range and that is why they show lower levels of conditioning. In this case, muscarinic agonists would be expected to further reduce conditioning in females, but antagonists would either have no effect or actually enhance conditioning, a trend we saw with scopolamine. Alternatively, males and females could have similar levels of muscarinic activation, but each sex requires a different level of activation for maximal contextual processing. Future experiments could differentiate these alternatives by performing complete dose response curves for the drugs administered here.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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