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ALINE KAUTZMANN SARTORI

O PAPEL DA OXITOCINA NO HIPOCAMPO DORSAL NA MEMÓRIA DE MEDO

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Trabalho de Conclusão de curso apresentado como requisito parcial para obtenção do título de Bacharel em Ciências Biológicas com ênfase em Neurociências na Universidade Federal do Rio Grande do Sul.

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Importância do estudo e contribuição pessoal para o desenvolvimento da pesquisa

Apesar de o hipocampo dorsal ser uma estrutura central na formação de memórias aversivas e integrar uma rede neural com regiões adjacentes também bastante importantes para a memória de medo (amígdala, córtex pré-frontal, etc), que inclusive já foram estudadas sob o efeito de oxitocina, o hipocampo em si é ainda uma estrutura pouco estudada neste meio; Além disso, a oxitocina possui papel de destaque, pois pode atuar tanto como neurotransmissor como hormônio no organismo, agindo em funções endócrinas, cognitivas e regulando o comportamento social. Já tem sido reportado vários resultados sob diferentes perspectivas no campo da memória, podendo ter possível papel relevante no tratamento de memórias patológicas em diferentes estruturas, porém, no hipocampo, tem poucos estudos envolvendo a ocitocina e explorando seu potencial efeito terapêutico. Dessa forma, este estudo tem sua importância porque visa investigar o papel da oxitocina no hipocampo dorsal, na região de CA1, na memória de medo, em ratos machos e fêmeas, e investigar seu potencial efeito benéfico para tratar memórias aversivas.

Eu participei integralmente do trabalho, contribuindo com a anestesia na cirurgia estereotáxica dos animais para colocação de cânulas, na parte comportamental com a manipulação e condicionamento dos ratos (incluindo os diferentes protocolos), e também nas infusões do fármaco ou veículo utilizando uma bomba de infusão acoplada a seringas Hamilton. Por fim, participei da análise dos resultados e da escrita do artigo.

Artigo

Oxytocin in the hippocampus impairs contextual fear memory formation in males and facilitates its attenuation: role in memory reactivation and extinction

Angel David Arellano Perez[1,2], Aline Kautzmann Sartori[1,3], Jordana Griebler Luft[1,2] and Lucas de Oliveira Alvares[1,2,*]

[1]Laboratório de Neurobiologia da Memória, Instituto de Biociências, Departamento de Biofísica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

[2]Programa da Pós-Graduação em Neurociências. Instituto de Ciências Básicas da Saúde (ICBS). Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

[3]Programa de Graduação em Ciências Biológicas, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

*Corresponding author: Lucas de Oliveira Alvares, Laboratório de Neurobiologia da Memória, Departamento de Biofísica. Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, Prédio 43422, sala 210, CEP 91.501-970, Porto Alegre, Brazil. E-mail: lucas.alvares@ufrgs.br.

Resumo

A formação de memórias é fundamental para nossa adaptação ao ambiente, mas dependendo do grau de aversão que uma determinada experiência simboliza, pode se tornar inconveniente e prejudicar gravemente a qualidade de vida do indivíduo, o que pode dar origem a transtornos psiquiátricos relacionados a traumas e estressores, como transtorno de estresse pós-traumático. A ocitocina é um neuropeptídeo envolvido na interação social, memória de reconhecimento social e, mais recentemente, na modulação da memória do medo. Esse hormônio tem sido estudado em memórias de medo em diferentes estruturas cerebrais, como córtex pré-frontal medial e amígdala basolateral, porém, ainda não foi estudado no hipocampo, apesar de ser uma estrutura central na consolidação de experiências aversivas. Diante disso, nosso objetivo é investigar o papel da ocitocina no hipocampo dorsal, na região CA1, nas diferentes fases da memória do medo, em ratos machos e fêmeas. Em nossos resultados, descobrimos que a ocitocina infundida no hipocampo após uma breve sessão de reativação da memória de medo atenua sua expressão em uma sessão de teste posterior.

Além disso, encontramos um prejuízo na aquisição da memória de medo sob o efeito da ocitocina em machos, mas não em fêmeas, e nenhum efeito na memória de extinção.

Abstract

The formation of memories is fundamental for our adaptation to the environment, but depending on the degree of aversion that a given experience symbolizes, it can become inconvenient and seriously impair the individual's quality of life, which can give rise to psychiatric disorders related to trauma and stressors such as post-traumatic stress disorder. Oxytocin is a neuropeptide involved in social interaction, social recognition memory, and more recently in the modulation of fear memory. This hormone has been studied in fear memories in different brain structures, such as the medial prefrontal cortex and basolateral amygdala, however, it has not yet been studied in the hippocampus, despite being a central structure in the consolidation of aversive experiences. Considering this, our objective is to investigate the role of oxytocin in the dorsal hippocampus, in the CA1 region, in the different phases of fear memory, in male and female rats. In our results, we found that oxytocin infused into the hippocampus after a brief fear memory reactivation session attenuates its expression in a later testing session. Furthermore, we found animpairment in the acquisition of fear memory under the effect of oxytocin in males, but not in females, and no effect on extinction memory.

Keywords

Oxytocin, Reactivation, Extinction, Females, Males, Hippocampus.

Highlights:

- *Fear memory acquisition in males, but not in females, is impaired by hippocampal administration of oxytocin.*
- *Oxytocin infused after a brief fear memory reactivation attenuates fear memory expression in a test session.*
- *Administration of oxytocin in the hippocampus showed no effect on extinction memory.*

1. Introduction

The formation of new memories is fundamental for our adaptation to the environment, but depending on the degree of aversion that a given experience symbolizes, it can becomes inconvenient and severely impair an individual's quality of

life, which can give rise to psychiatric disorders related to traumas and stressors, such as post-traumatic stress disorder (Parsons and Ressler, 2013). According to this information, a flexible mnemonic system becomes essential for us to know how to deal with the dynamism of everyday experiences and to constantly adapt to them.

In this line of interventions we have the reconsolidation mechanism, in which a previously consolidated memory can be modified during the period of destabilization induced by the reactivation of a memory (Nader et al., 2000), making it subject to changes both in its intensity and in its content (De Oliveira Álvares et al., 2013). We can also mention the extinction protocol, which is characterized by a long exposure of the subject to the trauma reminder in a place with the absence of the aversive stimulus, which favors the formation of a new memory that actively suppresses the original memory (Bouton, 2002) - however, this protocol has limitations: time and context dependence, in which extinction memory ceases to be expressed over time (spontaneous recovery) (Rescorla and Heth, 1975; Bouton et al., 2012), or when the subject is re-exposed to a reminder of the aversive event, which hinders the lasting attenuation of emotional fear responses when this protocol is used in therapeutic sessions during clinical practice. Alternatively, reconsolidation allows new information to be incorporated into the original memory trace (Alberini, 2011; Bonin and De Koninck, 2015; Nadel et al., 2012; Nader et al., 2000; Nader and Einarsson, 2010), updating it and allowing changes in its emotional content; in this way, this protocol becomes a more effective approach to attenuate in a lasting and resistant way the expression of a fear memory, since it does not have the limitations of extinction (Beckers and Kindt, 2017). However, it is also worth noting that several therapeutic strategies based on reconsolidation cannot be easily applied in clinical practice, since several drugs cannot be used in humans because they are toxic (Lee et al., 2006; Beckers and Kindt, 2017). Contrastingly, protocols that use positive valence stimuli such as chocolate, caffeine or methylphenidate in reactivation have also been documented in order to update the fear memory to a less aversive form (Haubrich et al., 2015; Pedraza et al., 2018; Arellano et al., 2020).

In this point of view, to recognize and treat pathological memories, it is also important to study the structures underlying to the formation and consolidation of fear memories, as well as knowledge about the role of neurotransmitters on them, such as oxytocin. Oxytocin is a cyclic neurohypophysis hormone that has a recognized role in social interaction and social recognition memory in different species (Hu et al., 2019;

Maroun and Wagner, 2016); more recently, its importance in the attenuation of fear and anxiety memory has been recognized in different brain structures such as medial prefrontal cortex and basolateral amygdala (Maroun and Wagner, 2016; Jang et al., 2022; Triana-Del Río, 2019). It is synthesized in the supraoptic and paraventricular nuclei of the hypothalamus and released in these areas in addition to the basolateral amygdala and the hippocampus (Hu et al., 2019, Le Dorze et al., 2020). This last structure, despite being central to the acquisition and consolidation of aversive memories and communicating with structures important for aversive memories such as the medial prefrontal cortex and the basolateral amygdala (Chaaya et al., 2018; Knierim, 2015), has been little studied under the effect of oxytocin on fear memories.

Considering that oxytocin has been shown to be important for modulating aversive memories in other brain structures, we propose to investigate the role of oxytocin in the dorsal hippocampus, in the CA1 region, in the different phases of fear memory, including consolidation, reconsolidation, and extinction (Matsushita et al., 2019; Zaninetti & Raggenbass, 2000).

2. Material and methods

2.1. Subjects

Were used male and female *Wistar* rats (2-3 months old, weighing approximately 300g) from CREAL at the Federal University of Rio Grande do Sul (UFRGS), which were housed 4 per cage in Plexiglas boxes, under a 12hs light/dark cycle (7 am/7 pm) at a constant controlled temperature (21ºC ±2), with water and food available *ad libitum.* All the procedures followed the Brazilian ethical guidelines for animal research.

2.2. Stereotaxic surgery, cannulae implantation and histology

Animals used in the experiment were anesthetized with ketamine and xylazine (75 and 10 mg/kg, respectively). Guide cannulae were bilaterally implanted at AP −4.2 mm (from bregma), LL ±3.0 mm, DV 1.8 mm, and 1.0 mm above the CA1 area of the dorsal hippocampus Behavioral procedures were performed 1 week after surgery. Animals with inaccurate cannulae positions were excluded from the statistical analysis.

The cannula position was verified at the end of each experiment. After euthanasia, the brain of the subjects was dissected and preserved in a solution of 4%

paraformaldehyde (PFA) for further analysis of cannulae placement in the target structure. Statistical analysis considered only animals with correct cannulae placements.

2.3. Drugs and microinfusion

Oxytocin (OXT) (syntocinon, Mylan Lab), is a peptide cyclic hormone of the posterior pituitary that plays an important role in social interaction (Maroun & Wagner, 2016). It was dissolved in saline solution (0.9% NaCl) to obtain a final concentration of 0.033 μg/μL or 0.07 μg/μL. Oxytocin 2μL (1 μL/side) or its vehicle (saline) was infused bilaterally into the CA1 region of the dorsal hippocampus 25 minutes prior to the conditioning session or immediately after using a pump connected to Hamilton syringes.

2.4. Contextual Fear Conditioning

The conditioning chamber for Contextual Fear Conditioning (CFC) training consisted of an illuminated plexiglass box (25 × 25 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1 cm apart). The training session was conducted in the light cycle, rats were placed in the chamber for 3 min, then received two foot-shocks 2s, 0.7 mA separated by a 30s interval, after 30 seconds of the last shock the animals were placed back in their home cage. A habituation session was conducted one day before conditioning training, all the rats were exposed to the conditioning chamber for 5 min.

2.4.1. Reactivation sessions

Reactivation sessions were performed 48 hours after training, in the same conditioning chamber without the presence of foot shocks. Fear memory was registered for 12 minutes (4 min block).

2.4.2. Extinction session

Extinction training consisted of a 30-minute session in the CAC, 48 hours after the training session. Fear memory was recorded in blocks of 5 minutes.

2.4.3. Test

The test session was performed in the same conditioning chamber for 5 minutes, one day after the reactivation session, or 2 hours after conditioning training (short-term memory).

2.5. Behavioral Measurement

Freezing behavior was used as a memory index, being recorded in real-time by an experienced observer blinded to the experimental conditions. Freezing is defined as the complete cessation of all movements except those necessary for respiration (Blanchard & Blanchard, 1969)

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2.6. Statistical Analysis

The statistical analyses were performed using the Student's *t-test*; one-way or two-way analysis of variance (ANOVA). Repeated measures ANOVA was used to analyze the reactivation time between the groups, and when necessary Tukey's *post-hoc* test was used. All data used the confidence level of 95% and the values of $P < 0.05$ were considered statistically significant. All experiments were randomized and performed blindly.

3. Results

3.1 Oxytocin impairs the acquisition of fear memory in males

It has been demonstrated that oxytocin has an important role in the consolidation of fear memories (Rasie Abdullahi, et al., 2018). However, its involvement in the hippocampus has been little studied. Here, we used two doses to evaluate the effect of oxytocin in the acquisition of fear memory in males. OXT (0.033 or 0.07, $n = 6$) or its vehicle ($n = 8$) was infused into hippocampus 25min previous CFC training. Test session was performed 48hrs later in the same context, without foot-shock presentation (fig. 1A).

We found that males receiving the 0.033 μg/μL dose showed impaired fear memory conditioning compared to the saline group and the 0.07 μg/μL dose (fig. 1B, one-way ANOVA, $F(2,17)=6.888$, $p=0.0064$; Tukey's *post-hoc*, saline vs OXT 0,033: $p=0.0095$; saline vs OXT 0,07: $p=0.9980$; OXT 0,033 vs OXT 0,07: $p=0.0168$). Based on this result, for the following experiments, we decided to use only the low dose of oxytocin (0,033u/l).

Since it has been shown that there is a difference in oxytocin receptor expression between males and females, it would be important to explore our findings in females as well (Dumais & Veenema, 2016). In this experiment we evaluated the effect of oxytocin

in the acquisition of fear memory in females, here, the same protocol described in figure 1 was performed. Oxytocin (*n*=9) or vehicle (*n*=8) was infused 25 minutes before training (fig. 1C). Interestingly, no effects were found in the session test (fig. 1D, Student's *t*-test, *t* (15) = 1.502, *p*= 0.1540). Our results show that oxytocin in the hippocampus has a negative impact on the acquisition of long-term fear memory, only in males.

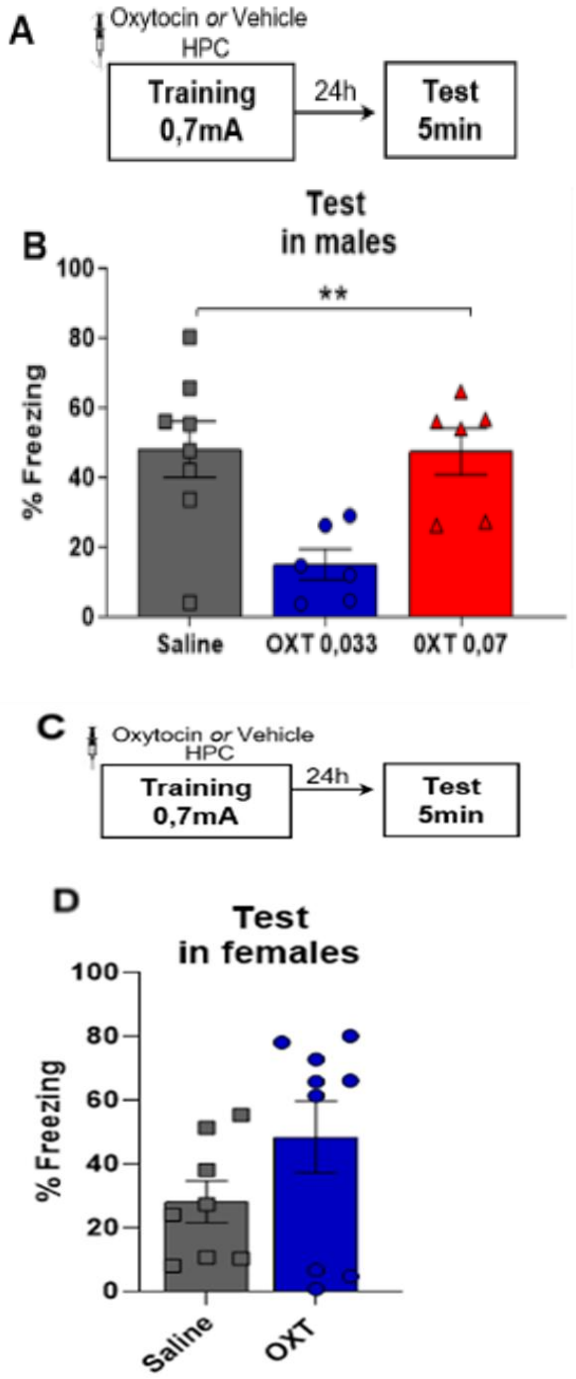

Figure 1. Oxytocin impairs fear memory acquisition in males. (A) Experimental design. Male rats were trained for CAC. OXT or your vehicle was infused in HPC 25 minutes prior to training. 24 hours later, a test session was performed. **(B)** Test session, OXT 0.033 expressed a lower freezing level compared to the control group and OXT 0.07. **(C)** Experimental design. Female rats were trained for CAC. OXT or your vehicle was infused in HPC 25 minutes prior to training. 24 hours later, a test session was performed. **(D)** Test session, no difference was observed between groups. Bars represent mean ± SEM. *p < 0.05.

3.2 *Oxytocin infusion into the hippocampus shows no effect in a fear memory extinction session*

The results of oxytocin administration in a fear memory extinction session are not entirely consistent, they may depend on the structure in which they are administered (Gunduz-Cinar et al., 2020). In this experiment, we aimed to evaluate the effect of oxytocin on the consolidation of extinction memory. Animals underwent the conditioning training described above and 48 hours later a fear memory extinction was performed with oxytocin (n=6) or vehicle (n=7) administered post session oxytocin to evaluate its participation in the fear memory extinction (fig. 3C). During the extinction session, repeated-measures ANOVA revealed no effect of time x group interaction ($F(5,25)=2.168$, $p=0.090$), nor significant effect between groups ($F(1,5)=0.476$, $p=0.521$). 24 hours later in a test session, no difference was found between the groups (fig. 3C, Student's t-test, t (15) = 0.6514, p= 0.5247). The results in this section show that oxytocin infused into the hippocampus after extinction training does not appear to be involved in extinction memory. Taken together, our results show that oxytocin in the hippocampus impairs the acquisition of fear memory in males, but in turn, facilitates its attenuation when infused after short memory reactivation, without effects on memory extinction.

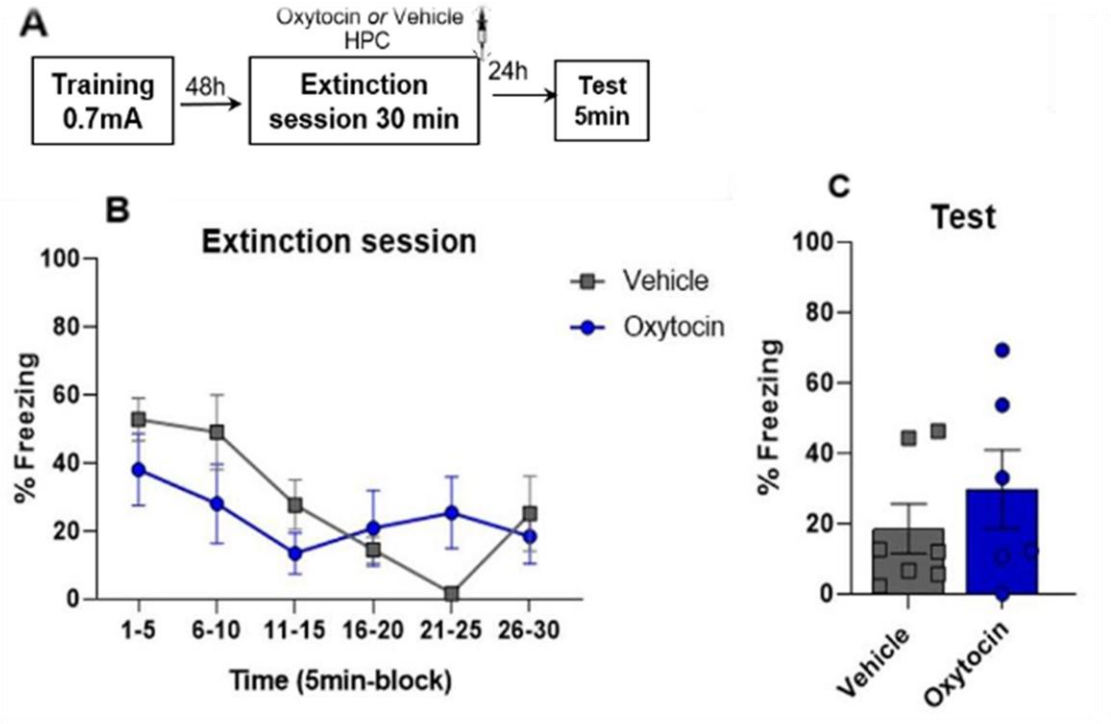

Figure 2. Oxytocin infusion into the hippocampus shows no effect on a fear memory extinction session. (A) Experimental design. Male rats were trained for CAC. 48 hours later they were taken for a 30-minute extinction session. OXT or your vehicle was infused with HPC immediately after training. 24 hours later, a test session was performed. **(B)** During the extinction session (expressed in 5-minute blocks), no difference was observed between groups and **(C)** Test session. Bars represent mean ± SEM. *p < 0.05.

3.3 Oxytocin attenuates the fear memory expression after memory reactivation

It has been demonstrated that oxytocin facilities the attenuation of fear memory (Guzmán et al., 2014). Here, we evaluated the effect of oxytocin in the hippocampus after a memory reactivation of 12 minutes. Animals were trained in CAC as described above and Oxytocin (n=8) or Vehicle (n=8) was infused into the hippocampus immediately after the reactivation session (fig. 2A). Repeated-measures ANOVA revealed no effect of time x group interaction (fig. 2B) ($F(2,10)=2.015$, p=0.184), nor significant effect between time (4 min blocks) ($F(2,10)=1.003$, p=0.401), or significant effect between groups ($F(1,5)=0.182$, p=0.688). Nevertheless, in the session test, the oxytocin group showed attenuation of fear memory compared to the control group (fig. 2C Student's t-test, t (10) = 2.482, p= 0.0324) showed differences in the freezing levels found in the test session.

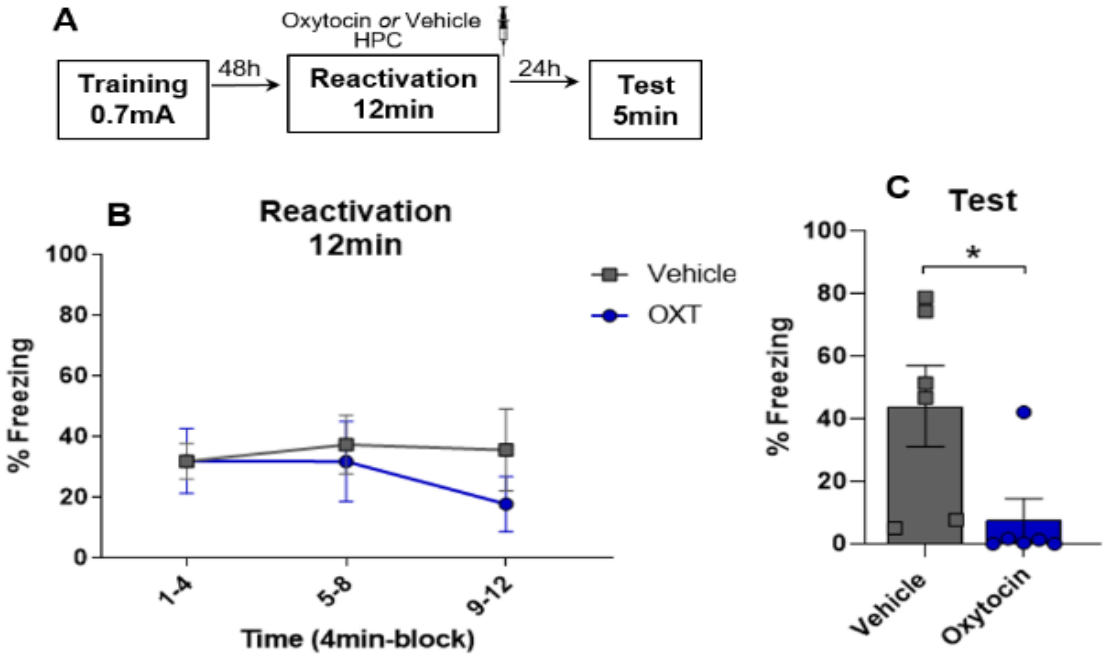

Figure 3. Oxytocin attenuates fear memory expression after memory reactivation. (A) Experimental design. Male rats were trained for CAC. 48h later they were taken for a 12min memory reset. OXT or your vehicle was infused with HPC immediately after the session. The next day, the test session was held. **(B)** During reactivation (expressed in 4-minute blocks), no difference was observed between groups. **(C)** Test session, the Oxytocin group expressed a lower freezing level compared to the control. Bars represent mean ± SEM. *p < 0.05.

4. Discussion

Here we have evaluated the effect of oxytocin in the hippocampus on the processes of formation, reactivation, and extinction of fear memories. Our results show that 0.033 µg/µL of oxytocin infused in the hippocampus before conditioning training generates an impairment in the acquisition of memory when tested the next day, particularly this condition was specific to males, in females no effects were found. Next, when OXT was administered immediately after a short memory reactivation session (12 minutes), we showed that in the test session the fear memory was attenuated (fig. 2). Finally, the effect of oxytocin was evaluated on the formation of memory extinction, no effects were found when oxytocin was administered after an extinction session (fig. 3).

Reports previously have demonstrated that oxytocin participates in the modulation of different cognitive processes including memory and learning (Wang et al., 2022). Most of them have studied the involvement of OXT in the prefrontal cortex, amygdala, and ICV (Janeček & Dabrowska, 2019). However, results can have different effects based on the structure where the drug is administrated and the doses (Olivera-

Pasilio & Dabrowska, 2020; Baldi et al., 2021). In the hippocampus, we have found only one work that was evaluating its effect on tone fear conditioning (Bazaz, et al., 2022), but in contextual fear memory, no studies have been reported to date. We believe that our results represent the first in this area. Here, we found that OXT prior to CFC impaired memory formation; this effect has also been demonstrated when OXT was infused via ICV prior to tone fear memory conditioning, generating a reduction in freezing in a subsequent session in male rats (Toth et al., 2012). Its administration into the amygdala also affects fear memory consolidation (Gunduz-Cinar et al., 2020; Campbell- Smith et al., 2015). These results support that OXT administration can impair the consolidation process. In another study, the application of atosiban via i.p (oxytocin antagonist) impaired fear memory consolidation, in fact, this effect was found only in long-term memory (Rasie Abdullahi et al., 2018). OXT is also involved during the release of the stress response, it participates in the down-regulation of the HPA axis response (Neumann et al., 2000: Wirth, 2015).

It is known that OXT exerts anti-stress effects by modulation of neural circuits in the hippocampus, amygdala, and prefrontal cortex (Matsushita et al., 2019). Here, we believe that OXT may also have an inverted U-shaped effect (Wirth, 2015) as HPA participates in the modulation of fear memory formation. Oxytocin showed to be necessary for consolidation, but its increase during acquisition may impair its formation. This U-shaped effect could be evaluated in future experiments. Another consideration is that oxytocin may influence the activity of a subclass of hippocampal GABAergic INs, increasing phasic and tonic GABAergic transmission in the CA1 region of the mouse hippocampus (Maniezzi et al., 2019). In rats, oxytocin is involved in the functional activity of excitatory circuits in the hippocampus by acting on inhibitory interneurons (Matsushita et al., 2019; Zaninetti & Raggenbass, 2000). We believe that this OXT activation on gabaergic activity would be interfering with the consolidation process. Surprisingly, no effect was found in females when OXT was administrated before the training session. Since oxytocin receptors are more present in females than males, we can explain this result based on sexual differences (Dumais & Veenema, 2016; Lu & Hu, 2021).

Our results also showed that after short memory reactivation, administration of OXT generated an attenuation of fear memory in the 12min reactivation. Here we believe that OXT would impair memory reconsolidation based on the decrease of the fear response in the session test. In fact, Hou et al., (2015) found that administration via

sc of oxytocin after a short reactivation impaired the reconsolidation showing a decrease in freezing. Our lab group has found previously that infusion of OXT in the hippocampus prior to memory reactivation facilitates attenuation of fear memory in the next test session (Arellano Perez et al., in construction). Different from consolidation, oxytocin perse appears not to be involved in the reconsolidation process. When atosiban was injected i.p. no effect was found after short memory reactivation (Rasie Abdullahi, et al., 2018). So, here we believe that OXT would be impairing the reconsolidation of memory by reducing its freezing level. Finally, on fear memories extinction, the results so far do not prove to be consistent, as described previously, via administration and concentration needs to be considered. In a recent study, OXT was infused (10 ng/0.5 µl) in the dorsal hippocampus and facilitated extinction to tone fear memory conditioning (Bazaz et al., 2022). Gunduz-Cinar et al. (2020) showed in a tone fear conditioning training that Intra-CeA OXT a dose of 1ng infused prior to extinction training interferences on expression memory and impairs recovery of fear memory extinction while a 0,01ug facilitated extinction memory, these effects were no found when administrated intra- BLA. In other study, OXT in CeA enhanced the expression of fear memory (0.6 to 75ng) and when administrated pre or post-extinction impairs the extinction; while in BLA facilitated extinction memory (Campbell-Smith et al., 2015). Toth et al. (2012) found also that microinfusion of OXT via ICV before extinction training resulted in enhanced freezing and impaired extinction in 0.1 and 1ug. In this work, no effects were found on extinction fear memory; we believe that OXT in the hippocampus may have a role in extinction memory based on the dose, unlike the amygdala, as previously reported. Since its effect is dose-dependent, future work needs to explore other doses into hippocampus. In summary, we conclude that oxytocin impairs fear memory acquisition in males (a) its infusion into the hippocampus after short memory reactivation attenuates fear memory expression (b) and no effects on extinction memory were found. These results found here should be considered as a function of sex and drug concentration.

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Competing interests

The authors declare no competing interests.

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