SOCIETÀ ITALIANA DI FARMACOLOGIA

RELAZIONE DI FINE PERIODO BORSA SIF-OTSUKA

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TITOLO DELLA RELAZIONE: Sex-specific effects of endocannabinoid modulation of fear memory dynamics

RELAZIONE:

Introduction

Fear memory extinction is an essential process in the ability to recover from highly stressful and traumatic events. Impaired fear extinction is believed to contribute to the development and persistence of psychiatric disorders, such as post-traumatic stress disorder (PTSD). Notably, only a small proportion of trauma exposed individuals develop PTSD, and women experience greater PTSD risk, prevalence, and duration than men (Breslau, 2009). The biological mechanisms underlying these sex differences, in terms of PTSD vulnerability and symptom severity in women, remain unclear and controversial. Yet, most studies on fear conditioning and extinction are exclusively performed in male subjects and studies comparing the sexes are few and inconsistent (Shansky, 2015). Some preclinical evidence describes a delay in the extinction of fear memory in females as compared to males, while other studies report that females demonstrate higher extinction rates than males (Shansky, 2015). It has recently been shown that these effects did not necessarily reflect alterations in fear memory extinction learning, but were rather related to sex differences in fear response strategies (Gruene *et al*, 2015a).

In the laboratory, neural mechanisms underlying associative fear learning and fear memory extinction are usually examined by using the classical Pavlovian auditory or cued fear conditioning

paradigm. In the conditioning phase of an auditory fear conditioning paradigm, a footshock (unconditioned stimulus) is associated with a tone (conditioned stimulus) following repeated presentations of the pair. During extinction training a safe memory is developed, and the aversive memory is generally overpowered by repeated presentations of the tone in the absence of the shock (LeDoux, 2000; Maren, 2001). In this behavioral paradigm, the memory strength is assessed by quantifying the time during which the animals exhibit freezing behavior, a form of passive fear response, defined as the absence of movements except those needed for respiration (Fanselow, 1980). These studies have mostly been conducted in male subjects which predominately express freezing behavior. Conversely, a substantial proportion of female rats exhibit low freezing levels during fear conditioning and extinction, and express learned fear-engaged darting behavior, a rapid, forward movement that resembles an active and escape-like fear response (Gruene *et al*, 2015a).

The endocannabinoid system – composed of the cannabinoid type 1 and 2 receptors (CB1R and CB2R), two main endogenous ligands anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and their respective hydrolyzing enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) – is heavily involved in modulating fear memory extinction (Gunduz-Cinar *et al*, 2013; Marsicano *et al*, 2002; Morena *et al*, 2016). Moreover, preclinical findings from male animals have shown that the recruitment of active and passive fear coping responses depends on amygdala cannabinoid signaling, wherein CB1R, seems to be necessary for the temporal shift between passive (predominant at the beginning of extinction) and active (increasingly exploratory and predominant at the end of extinction) coping strategies (Metna-Laurent *et al*, 2012). However, to date little is known about whether endocannabinoids regulate fear memory and fear coping strategies in females. Therefore, the current study focuses on the examination of potential sex differences in endocannabinoid signaling in rats during auditory fear memory extinction, in brain regions importantly involved in fear memory and fear expression (amygdala, prefrontal cortex, hippocampus, and periaqueductal gray) (Corcoran and Maren, 2004; Farook *et al*, 2004; Gruene *et al*, 2015b; Sierra-Mercado *et al*, 2011; Vianna *et al*, 2001; Watson *et al*, 2016). These findings are integrated with behavioral pharmacology to determine whether pharmacological manipulations of the endocannabinoid system modulate fear memory extinction and fear coping responses in male and female rats.

Materials and methods

Male and female adult Sprague Dawley rats (300-400 g at the time of testing; Charles River Laboratories) were tested in an auditory fear conditioning paradigm. Rats were handled for 1min each, for three consecutive days (day 1, day 2 and day 3) before fear conditioning. On day 2 and 3, immediately after the handling procedure animals were habituated to the fear-conditioning chamber (context A - grid floor, back and side metal walls, clear Plexiglas front door and ceiling, and white light; day 2) and to the fear extinction chamber (context B – white opaque plastic floor and walls and white light; day 3) for 10 minutes. On day 4, fear conditioning was performed in context A. After a 5-minute acclimation period, all rats were exposed to seven conditioning trials. Each conditioning trial involved presentation of the conditioned stimulus (CS; 80dB, 4Hz tone) for 30 seconds, co-terminating with a 1 second unconditioned stimulus (US; 0.65mA shock). Inter-trial interval (ITI) between two consecutive CS-US pairings was 3 minutes. After conditioning, each rat was returned to its home cage. On day 5, extinction training was conducted in context B. After a 2-minute acclimation period, 20 x CS presentations were delivered (2-minute inter-CS interval). To

evaluate whether memory extinction had occurred (extinction retrieval), on day 6, animals were placed in context B and presented with 5 CSs (2-min inter-CS interval).

To examine the role of the endocannabinoid system in modulating fear extinction dynamics, 1 hour before extinction training, male and female rats were intraperitoneally (i.p.) injected with the FAAH inhibitor URB597 (0.3 mg/kg), the MAGL inhibitor MJN110 (10 mg/kg) or their vehicle (5% polyethylene glycol, 5% Tween80, 90% saline). To evaluate whether the effects of URB597 or MJN110 were mediated by the activation of CB1R, separate groups of rats were injected with the CB1R antagonist AM251 (1 mg/kg, i.p.) alone or in combination with URB597 or MJN110, 30 min before URB597 or MJN110 injections.

In a separate experiment, two cohorts of animals (Extinction and No-Extinction groups) which did not receive any pharmacological treatment, AEA and 2-AG levels were measured in the amygdala, prefrontal cortex, hippocampus, and periaqueductal gray immediately after the extinction training session. For the Extinction group, fear conditioning and extinction training were conducted as described above. The No-Extinction control group received regular fear conditioning, but, on the extinction training (day 5), was exposed to context B for an equivalent amount of time without tone presentations. Immediately after the extinction training session, rats were sacrificed and brain regions were rapidly dissected, frozen on dry ice and stored at -80°C. The lipid extraction process was performed as described before (Morena *et al*, 2015). Brain tissues were weighed and homogenized with glass rods in borosilicate class culture tubes containing 2 ml acetonitrile, 5 pmol of [2H8] AEA, and 5 nmol of [2H8] 2-AG. Following 20 min tissue sonication in ice water, samples were incubated overnight at -20°C to allow the precipitation of proteins. Samples were then centrifuged at 1500 x g to separate supernatants, which was then transferred to a subsequent glass tube and evaporated to dryness in the presence of N_2 gas. A volume of 300 µL acetonitrile was added to the dried samples, and then dried again in the presence of N_2 gas. AEA and 2-AG lipid extracts were suspended in 20 µL acetonitrile and stored at -80°C until liquid chromatography mass spectrometry analysis as previously detailed (Qi *et al*, 2015).

Results and Conclusion

Overall, accordingly with Gruene *et al,* 2015a, our results show a clear sexual dimorphism in fear coping responses during the auditory fear conditioning paradigm. While males predominantly froze, females expressed lower freezing and greater darting as compared to males throughout each experimental stage. Interestingly, our pharmacological manipulations revealed that 0.3 mg/kg URB597, administered systemically 1 hour prior to extinction training trended towards greater freezing and attenuated darting in females. Furthermore, we found that these effects were not mediated by CB1R, as treatment with the CB1R antagonist AM251 did not block URB597-induced increasing in freezing behavior in females. Of note, freezing behavior during the extinction training session further increased when female rats were administered AM251 alone or, to a greater extent, when females were injected AM251 in combination with URB597. The AM251+URB597 effect on freezing behavior persisted into the subsequent day (extinction retrieval), where females for this group showed significantly higher freezing than all other groups, suggesting weakened memory extinction.
Besides CB1R, AEA is known to activate transient receptor potential vanilloid type-1 channels (TRPV1) (Di Marzo and De Petrocellis, 2012; Zygmunt *et al*, 1999), thus it is possible that the

potentiated freezing observed in females threated with AM251 or AM251 together with URB597, might be the outcome of CB1R antagonism facilitating robust AEA-mediated TRPV1 activation. Indeed, TRPV1 has been suggested to facilitate freezing expression and TRPV1 KO mice have been reported to show reduced anxiety and diminished fear behavior compared with their wild-type littermates (Marsch *et al*, 2007).

Concerning MJN110 treatment, our preliminary data show that boosting 2-AG levels induced a trend towards lower freezing and greater darting in females, which was totally abolished with AM251 administration, thus suggesting that 2-AG, by means of the CB1R, enhances the natural fear coping strategies of females (i.e., enhancing darting while attenuating freezing).

Conversely, neither URB597 nor MJN110 treatments, at least at the doses we used, altered fear memory or fear coping behavior in male rats.

Interestingly, data collected so far, show that, among control groups, females presented increased amygdala AEA content as compared to males and no sex differences were observed in 2-AG levels. However, when considering the effect of memory extinction in females, we did not find any difference neither in AEA nor 2-AG levels between rats that did undergo extinction compared to rats that were only exposed to the context but never presented with the aversive tone (No-Extinction group). Of note, accordingly with our behavioral results, average female darting was positively correlated with amygdala 2-AG content, thus suggesting that increased 2-AG signaling in the amygdala may promote active fear coping behavior.

According with previous findings (Gunduz-Cinar *et al*, 2013; Marsicano *et al*, 2002), we found increased amygdala AEA content in the male Extinction group compared to the male No-Extinction group. The extinction procedure did not affect amygdala 2-AG levels in males.

Endocannabinoid level measurement from other brain regions collected (hippocampus, prefrontal cortex and periaqueductal gray) will supplement the present findings.

In agreement with our behavioral results in females, it is interesting to note that preclinical findings from male mice have shown that boosting AEA signaling increases passive and reduces active fear coping strategies, while increased 2-AG signaling promotes active fear responses (Heinz *et al*, 2017). Conversely, in an auditory fear conditioning study, albeit with a different protocol as ours, boosting 2-AG in male mice impaired within-session extinction (i.e., resulting in persistently heightened freezing) soon after conditioning, an effect which was mediated by CB1R (Hartley *et al*, 2016). Increased AEA, both systemically or directly within the amygdala, has been shown to facilitate fear extinction through CB1R activation in male mice (Gunduz-Cinar *et al*, 2013). Differences between these studies and ours may arise from alternative fear conditioning paradigms used, different drugs or doses, and that we used rats versus mice.

In conclusion, our preliminary results reinforce the sex-different fear coping behaviors demonstrated in an aversive conditioning paradigm (Gruene *et al*, 2015a). Our findings support a role of AEA in enhancing passive, and diminishing active, fear responses, and impairing fear memory extinction in females; these effects, are likely mediated by TRPV1 activation. Furthermore, our data suggest that increased 2-AG signaling diminishes passive, and increases active, fear responses in females, through CB1R activation.

As memory extinction is relevant to the pathogenesis of PTSD, which disproportionately affects women (Breslau, 2009), identification of the neurobiological mechanisms underlying sex differences in fear memory extinction and fear coping strategies will facilitate the development of sex-specific approaches for the treatment of stress-related disorders. Targeting the endocannabinoid system to

treat PTSD is currently being examined at a clinical level, these data may help to elucidate the mechanisms by which these agents exert therapeutic benefit and provide a greater understanding of the mechanisms underlying the sexually dimorphic effects of cannabinoids.

References

Breslau N (2009). The Epidemiology of Trauma, PTSD, and Other Posttrauma Disorders. *Trauma, Violence, Abus* **10**: 198–210.

Corcoran KA, Maren S (2004). Factors regulating the effects of hippocampal inactivation on renewal of conditional fear after extinction. *Learn Mem* **11**: 598–603.

Fanselow MS (1980). Conditional and unconditional components of post-shock freezing. *Pavlov J Biol Sci Off J Pavlov* **15**: 177–182.

Farook JM, Wang Q, Moochhala SM, Zhu ZY, Lee L, Wong PT-H (2004). Distinct regions of periaqueductal gray (PAG) are involved in freezing behavior in hooded PVG rats on the cat-freezing test apparatus. *Neurosci Lett* **354**: 139–42.

Gruene TM, Flick K, Stefano A, Shea SD, Shansky RM (2015a). Sexually divergent expression of active and passive conditioned fear responses in rats. *Elife* **4**: 1-9.

Gruene TM, Roberts E, Thomas V, Ronzio A, Shansky RM (2015b). Sex-specific neuroanatomical correlates of fear expression in prefrontal-amygdala circuits. *Biol Psychiatry* **78**: 186–93.

Gunduz-Cinar O, MacPherson KP, Cinar R, Gamble-George J, Sugden K, Williams B, *et al* (2013). Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity. *Mol Psychiatry* **18**: 813–23.

Hartley ND, Gunduz-Cinar O, Halladay L, Bukalo O, Holmes A, Patel S (2016). 2-arachidonoylglycerol signaling impairs short-term fear extinction. *Transl Psychiatry* **6**: e749.

Heinz DE, Genewsky A, Wotjak CT (2017). Enhanced anandamide signaling reduces flight behavior elicited by an approaching robo-beetle. *Neuropharmacology* **126**: 233–241.

LeDoux JE (2000). Emotion Circuits in the Brain. *Annu Rev Neurosci* **23**: 155–184.

Maren S (2001). Neurobiology of Pavlovian Fear Conditioning. *Annu Rev Neurosci* **24**: 897–931.

Marsch R, Foeller E, Rammes G, Bunck M, Kössl M, Holsboer F, *et al* (2007). Reduced anxiety, conditioned fear, and hippocampal long-term potentiation in transient receptor potential vanilloid type 1 receptor-deficient mice. *J Neurosci* **27**: 832–9.

Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, *et al* (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature* **418**: 530–534.

Marzo V Di, Petrocellis L De (2012). Why do cannabinoid receptors have more than one endogenous ligand? *Philos Trans R Soc Lond B Biol Sci* **367**: 3216–28.

Metna-Laurent M, Soria-Gómez E, Verrier D, Conforzi M, Jégo P, Lafenêtre P, *et al* (2012). Bimodal control of fear-coping strategies by CB_1 cannabinoid receptors. *J Neurosci* **32**: 7109–18.

Morena M, Castro V De, Gray JM, Palmery M, Trezza V, Roozendaal B, *et al* (2015). Training-Associated Emotional Arousal Shapes Endocannabinoid Modulation of Spatial Memory Retrieval in Rats. *J Neurosci* **35**: 13962–74.

Morena M, Patel S, Bains JS, Hill MN (2016). Neurobiological Interactions Between Stress and the Endocannabinoid System. *Neuropsychopharmacology* **41**: 80–102.

Qi M, Morena M, Vecchiarelli HA, Hill MN, Schriemer DC (2015). A robust capillary liquid chromatography/tandem mass spectrometry method for quantitation of neuromodulatory endocannabinoids. *Rapid Commun Mass Spectrom* **29**: 1889–1897.

Shansky RM (2015). Sex differences in PTSD resilience and susceptibility: Challenges for animal models of fear learning. *Neurobiol Stress* **1**: 60–65.

Sierra-Mercado D, Padilla-Coreano N, Quirk GJ (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology* **36**: 529–38.

Vianna DM, Graeff FG, Landeira-Fernandez J, Brandão ML (2001). Lesion of the ventral periaqueductal gray reduces conditioned fear but does not change freezing induced by stimulation of the dorsal periaqueductal gray. *Learn Mem* **8**: 164–9.

Watson TC, Cerminara NL, Lumb BM, Apps R (2016). Neural Correlates of Fear in the Periaqueductal Gray. *J Neurosci* **36**: 12707–12719.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgård M, Marzo V Di, *et al* (1999). Vanilloid

receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**: 452–7.

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