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A DISSERTATION IN FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY IN TOXICOLOGY

RESILIENCE AND SUSCEPTIBILITY TO STRESS-RELATED DISORDERS: INSIGHTS FROM ANIMAL MODELS

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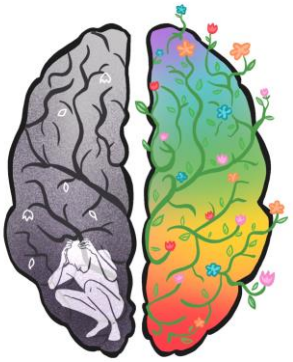
To my self

“It’s not stress that kills us, it is our reaction to it”

(Hans Selye)

CONTENTS

GENERAL INTRODUCTION



Exposure to stress can lead to the development of stress-related diseases, such as anxiety, depression and post-traumatic stress disorder (PTSD) (De Kloet et al., 2005; McEwen, 2003). However, experiencing stressful events do not affect everyone similarly. It is well known that stress response cannot be sustained for a long time, the organism thus needs to develop effective physiological and psychological changes to cope with it (McEwen, 2007). Stressful experiences lead indeed to adaptation, but in some susceptible individuals this can be dysfunctional (Del Giudice et al., 2011; Ullmann et al., 2019). Compelling evidence demonstrated that stressful experiences produce long-lasting memories through the activation of several physiological responses which end with stress mediators release (McGaugh, 2013; Roozendaal and McGaugh, 2011). However, if on one side memories can be strengthened by stress or emotional arousing situations, on the other side they could undergo different modifications (Schacter, 1999). In fact, stressful experiences can produce both accurate or generalized memories, reflecting an interindividual difference in response to stress (Bahtiyar et al., 2020; Kalisch et al., 2017; Wu et al., 2013). It has long been observed in humans that drugs of abuse affect memory processes (Goodman and Packard, 2016; Kutlu and Gould, 2016), but their effects on memory generalization are poorly investigated. From an ethological perspective, memory generalization may be both adaptive and maladaptive (Asok et al., 2019). For example, the excessive memory retrieval of a fear related situation in a context with high predatory risk, is a common adaptive response in animals, but this excessive memory retrieval in an environment lacking of an imminent threat is maladaptive and represents the core of fear memory generalization (Asok et al., 2019). The overgeneralization of a contextual fear memory is one of the hallmark symptoms of many anxiety and stress-related disorders, such as PTSD (Dunsmoor and Paz, 2015; Elzinga and Bremner, 2002; Lissek and Grillon, 2010).

Evidence exists that the anesthetic ketamine, also known for its abuse potential, modulates or aggravates early post-traumatic stress reactions when given in the acute trauma phase, and it correlates with the onset of sustained PTSD symptomatology

(Schönenberg et al., 2008, 2005; Winter and Irle, 2004). Conversely, recent findings indicate that ketamine, at sub-anesthetic doses, improves psychiatric symptoms in individuals already suffering from PTSD and comorbid depression (Albott et al., 2018; Hartberg et al., 2018). This suggests that ketamine might increase the risk of PTSD development when given in close proximity of the trauma, plausibly by potentiating the noradrenergic-mediated enhancement of memory consolidation. However, the specific mechanisms underlying these ketamine-induced effects have not been clarified yet. PTSD is a chronic psychiatric disease characterized by increased anxiety, altered sociability and cognitive alterations (e.g. over-consolidation, memory generalization and impaired extinction of the trauma) (American Psychiatric Association, 2013; Yehuda et al., 2015). Since the ability to examine the neurobiological underpinnings of PTSD in humans is limited, preclinical models represent a valuable tool to explore the long-term consequences of trauma exposure (Berardi et al., 2014; Richter-Levin, 1998; Verbitsky et al., 2020; Zoladz and Diamond, 2016).

The single prolonged stress paradigm is a valid model to resemble the core symptoms of PTSD (Yamamoto et al., 2009). The manifestation of a PTSD-like phenotype following SPS exposure is time-dependent and requires 7-14 days of incubation (i.e. sensitization or consolidation period) to develop (Knox et al., 2012; Liberzon et al., 1999; Wu et al., 2016). Thus, in most of the studies investigating the neurobiology of PTSD, rodents subjected to a single prolonged stress protocol are left undisturbed for this time window and tested 7-14 days after trauma exposure (Liberzon et al., 1999; Yamamoto et al., 2009). Given that PTSD is a chronic psychiatric disease, it is of critical importance to evaluate whether the effects induced by single prolonged stress are present long after stress exposure (Sillivan et al., 2017).

Beyond the chronicity aspect of PTSD, another important issue of this pathology is the interindividual variability in response to trauma. Almost all PTSD experimental models homogenize all the trauma-exposed animals as having the same phenotype, regardless of the susceptibility or resilience to develop a PTSD-like phenotype, thus lacking of construct and predictive validity (Holly and Miczek, 2015). Moreover, in the spare

studies that consider the individual variability to trauma, only the anxiety symptoms are used to discern between different PTSD-like phenotypes (Musazzi et al., 2018; Ritov et al., 2016), without considering the PTSD etiopathogenesis (e.g. cognitive alterations such as the excessive memory consolidation and retrieval as well as the impaired extinction). On the contrary, in the clinical scenario literature data demonstrate that even if the vast majority of individuals will experience a trauma once throughout their life, only a small subset of them develops PTSD while the others fully recover and mostly without any specific intervention (Javidi and Yadollahie, 2012). This evidence highlights the existence of different trauma response phenotypes among the human population, and that genetic, epigenetic and environmental factors can play a crucial role in the susceptibility to PTSD development determining how the stress response to the traumatic experience can lead to aberrant and enduring alteration of memory processes (Bolsinger et al., 2018; Skelton et al., 2012; Yehuda et al., 2015).

Among the environmental factors involved in the development of psychiatric diseases, experiencing stressful events, especially during the early life, may lead to coping strategies which in turn may confer resilience or vulnerability to later life stressful events and the subsequent development of stress-related psychopathologies (Daskalakis et al., 2013). To date, human data reporting the influence of experiencing bullying during adolescence on the development of psychiatric disorders and its seriousness after an additional trauma in adulthood, are lacking. Bullying and subordination episodes are social stressors that frequently occur among adolescents (Menesini and Salmivalli, 2017; Rettew and Pawlowski, 2016). Clinical evidence has demonstrated that adolescent bully-victims are more predisposed to develop psychiatric diseases later in life, in particular anxiety, depression and PTSD (Gladstone et al., 2006). Because adolescence is a crucial developmental stage associated with profound changes in the structure and function of the brain (Casey et al., 2008; Romeo and McEwen, 2006; Spear, 2000a), stress experienced during this critical developmental period has more detrimental effects compared with those experienced in adulthood.

Adolescence is characterized by elevated social interactions, play behaviors and

novelty-seeking, all behavioral features necessary to acquire the skills for autonomy and independence from parental caretakers (Spear, 2000b). Thus, the deprivation of social interactions with conspecifics during this specific life period may produce deleterious effects which long-term persist in rodents (Fone and Porkess, 2008; Mumtaz et al., 2018). Social isolation stress is the most commonly used stress paradigm to reproduce in rodents several neurochemical and behavioral alterations resembling some of the core symptoms observed in schizophrenic patients (Fone and Porkess, 2008; Heidbreder et al., 2000). Generally, it is performed for long and continuative periods (e.g. 4-6 weeks or more) from weaning to adulthood (Heidbreder et al., 2000; Lapiz et al., 2003). However, nowadays understanding the effects of brief periods of social isolation stress is more and more becoming an impelling issue. Indeed, spending daily brief and repeated periods on social platforms or playing video games has become a habit among the current generation of the adolescents and when it became chronic, it could represent a serious public health problem (Anderson et al., 2010; Müller and Wölfling, 2017; Von Der Heiden et al., 2019).

Outline

Stressful experiences can produce both accurate or generalized memories, reflecting an interindividual difference in response to stress (Bahtiyar et al., 2020; Kalisch et al., 2017; Wu et al., 2013). Psychostimulants outcomes on memory enhancement are known from many years, but literature data also show their memory generalization effects (Ballard et al., 2012; Easton and Bauer, 1997). However, the mechanisms through which psychostimulants affect memory quality is still poor investigated. In **Chapter 1** we explored the memory generalization effects induced by amphetamine and a new psychostimulant: the 3,4-methylenedioxypyrovalerone (MDPV), also called “bath salt”. Both psychostimulants share a similar, yet not identical mechanism of action, augmenting noradrenaline and dopamine levels in the synaptic cleft. Thus, in a second experiment we aimed at evaluating the different involvement of the

noradrenergic and dopaminergic system in the effects on memory enhancement, and on memory generalization.

Treatment with the anesthetic ketamine, a renowned drug of abuse, in trauma patients during emergency care aggravated early post-traumatic stress reaction which is highly predictive of PTSD development and severity (Schönenberg et al., 2008, 2005; Winter and Irle, 2004). Based on the evidence that ketamine induces a robust central and peripheral adrenergic/noradrenergic potentiation and that activation of this system is essential for the formation of memory for stressful events, in **Chapter 2** we explored the possibility that the strong sympathomimetic action of ketamine might underlie its memory enhancing effects.

Given that PTSD is a chronic psychiatric disease, it is of critical importance to evaluate whether animal model of PTSD resemble the chronicity nature of this pathology. The single prolonged stress paradigm has been extensively shown to induce behavioral and endocrine effects resembling the hallmark symptoms observed in PTSD patients (Lisieski et al., 2018; Souza et al., 2017; Verbitsky et al., 2020; Yamamoto et al., 2009) and, similarly to PTSD, the manifestation of these effects is time-dependent and requires a 1-2 weeks incubation period (Knox et al., 2012; Liberzon et al., 1999; Wu et al., 2016). Although women have a two-fold greater risk to develop PTSD, most preclinical studies have been carried out in males. In **Chapter 3** we aimed at investigating whether SPS induced persistent PTSD-like behavioral alterations in rats long after trauma exposure and whether these effects are sex-dependent.

Another important aspect of PTSD is the susceptibility to develop this pathology. Animal models are a useful tool to investigate this issue. However, in the sparse studies considering the individual variability in response to trauma, only the anxiety symptoms are used to discern between different PTSD-like phenotypes (Musazzi et

al., 2018; Ritov et al., 2016), without considering the cognitive aspects of PTSD. In **Chapter 4** we aimed at the development of an animal model of PTSD, with translational value, able to predict the susceptibility and the resilience phenotype considering both the cognitive and emotional alterations long after trauma. For this purpose, we outstretched our previously validated animal model (Berardi et al., 2014) in order to identify susceptible and resilient rats in terms of over- consolidation, impaired extinction and social behavior alterations.

Nowadays there are different hypotheses to explain the interindividual variability in response to stress. Different studies used the "two hit" stress model to investigate whether exposure to two different stressors at different ages may increase (or decrease) the risk to develop psychopathologies after experiencing the second stressor (Hill et al., 2014; Peña et al., 2017; Tsoory et al., 2007). However, how a social stress similar to bullying in humans experienced at adolescence may affect the reaction to additional stressor later in life are less investigated. Adolescence is a period of impressive brain maturation in which the structure of the brain is ever-changing (Spear, 2000). Thus, maintaining a correct balance between mediators that sustain synaptic plasticity is of utmost importance. In **Chapter 5** we evaluated whether the exposure to social defeat stress, a highly validated animal model of bullying in rodents, at adolescence and/or single prolonged stress experienced at adulthood affect the later development of emotional and cognitive alterations and whether the behavioral alterations are linked to any modification of hippocampal brain derived neurotrophic factor (BDNF) expression and plasma corticosterone levels.

While adolescent bullying occurs in a small subset of population, a more common social stressor is represented by the deprivation of social interactions with conspecifics which is able to induce profound behavioral changes in rodents (Fone and Porkess, 2008; Heidbreder et al., 2000). Social isolation stress paradigm is commonly used to

reproduce schizophrenia in rodents and it is conducted chronically from weaning to adulthood (Fone and Porkess, 2008; Mumtaz et al., 2018). However, long-term effects induced by a briefer period of social isolation stress during adolescence, which is a critical window for brain development, are not investigated. Moreover, it is known that in mammals, females have greater risk to develop stress-related disorders than males (Heck and Handa, 2019; McCormick and Mathews, 2007). In **Chapter 6** we firstly evaluated whether repeated brief periods of social isolation stress may alter emotionality and cognitive functions in adult male rats. Secondly, we examined whether brief and repeated social isolation stress during at adolescence and/or single prolonged stress at adulthood affect the later development of alteration on emotionality and cognition. Further, we aimed at evaluating whether such effects are sex-dependent.

Chapter 7 summarizes and discusses the findings of this thesis and provides conclusions and future perspectives.

References

Albott, C.S., Lim, K.O., Forbes, M.K., Erbes, C., Tye, S.J., Grabowski, J.G., Thuras, P., Batres-Y-Carr, T.M., Wels, J., Shiroma, P.R., 2018. Efficacy, Safety, and Durability of Repeated Ketamine Infusions for Comorbid Posttraumatic Stress Disorder and Treatment-Resistant Depression. J. Clin. Psychiatry 79, pii: 17m11634. https://doi.org/10.4088/JCP.17m11634

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association. https://doi.org/10.1176/appi.books.9780890425596

Anderson, C.A., Shibuya, A., Ihori, N., Swing, E.L., Bushman, B.J., Sakamoto, A., Rothstein, H.R., Saleem, M., 2010. Violent Video Game Effects on Aggression, Empathy, and Prosocial Behavior in Eastern and Western Countries: A Meta-Analytic Review. Psychol. Bull. https://doi.org/10.1037/a0018251

Asok, A., Kandel, E.R., Rayman, J.B., 2019. The neurobiology of fear generalization. Front. Behav. Neurosci. https://doi.org/10.3389/fnbeh.2018.00329

Bahtiyar, S., Gulmez Karaca, K., Henckens, M.J.A.G., Roozendaal, B., 2020. Norepinephrine and glucocorticoid effects on the brain mechanisms underlying memory accuracy and generalization. Mol. Cell. Neurosci. https://doi.org/10.1016/j.mcn.2020.103537

Ballard, M.E., Gallo, D.A., De Wit, H., 2012. Psychoactive drugs and false memory: Comparison of dextroamphetamine and delta-9-tetrahydrocannabinol on false recognition. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-011-2374-5

Bahtiyar, S., Gulmez Karaca, K., Henckens, M.J.A.G., Roozendaal, B., 2020. Norepinephrine and glucocorticoid effects on the brain mechanisms underlying memory accuracy and generalization. Mol. Cell. Neurosci. https://doi.org/10.1016/j.mcn.2020.103537

Berardi, A., Trezza, V., Palmery, M., Trabace, L., Cuomo, V., Campolongo, P., 2014. An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD). Front. Behav. Neurosci. 8. https://doi.org/10.3389/fnbeh.2014.00142

Bolsinger, J., Seifritz, E., Kleim, B., Manoliu, A., 2018. Neuroimaging Correlates of Resilience to Traumatic Events—A Comprehensive Review. Front. Psychiatry. https://doi.org/10.3389/fpsyt.2018.00693

Casey, B.J., Jones, R.M., Hare, T.A., 2008. The adolescent brain. Ann. N. Y. Acad. Sci. https://doi.org/10.1196/annals.1440.010

Daskalakis, N.P., Bagot, R.C., Parker, K.J., Vinkers, C.H., de Kloet, E.R., 2013. The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome. Psychoneuroendocrinology 38, 1858–1873. https://doi.org/10.1016/j.psyneuen.2013.06.008

De Kloet, E.R., Joëls, M., Holsboer, F., 2005. Stress and the brain: From adaptation to disease. Nat. Rev. Neurosci. https://doi.org/10.1038/nrn1683

Del Giudice, M., Ellis, B.J., Shirtcliff, E.A., 2011. The Adaptive Calibration Model of stress responsivity. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2010.11.007

Dunsmoor, J.E., Paz, R., 2015. Fear Generalization and Anxiety: Behavioral and Neural Mechanisms. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2015.04.010

Easton, C.J., Bauer, L.O., 1997. Beneficial effects of thiamine on recognition memory and P300 in abstinent cocaine-dependent patients. Psychiatry Res. https://doi.org/10.1016/S0165-1781(97)00024-3

Elzinga, B.M., Bremner, J.D., 2002. Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)? J. Affect. Disord. https://doi.org/10.1016/S0165-0327(01)00351-2

Fone, K.C.F., Porkess, M.V., 2008. Behavioural and neurochemical effects of post-weaning social isolation in rodents-Relevance to developmental neuropsychiatric disorders. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2008.03.003

Gladstone, G.L., Parker, G.B., Malhi, G.S., 2006. Do Bullied Children Become Anxious and Depressed Adults? J. Nerv. Ment. Dis. 194, 201–208. https://doi.org/10.1097/01.nmd.0000202491.99719.c3

Goodman, J., Packard, M.G., 2016. Memory systems and the addicted brain. Front. Psychiatry. https://doi.org/10.3389/fpsyt.2016.00024

Hartberg, J., Garrett-Walcott, S., De Gioannis, A., 2018. Impact of oral ketamine augmentation on hospital admissions in treatment-resistant depression and PTSD: a retrospective study. Psychopharmacology (Berl). 235, 393–398. https://doi.org/10.1007/s00213-017-4786-3

Heidbreder, C.A., Weiss, I.C., Domeney, A.M., Pryce, C., Homberg, J., Hedou, G., Feldon, J., Moran, M.C., Nelson, P., 2000. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neuroscience. https://doi.org/10.1016/S0306-4522(00)00336-5

Hill, R.A., Kiss Von Soly, S., Ratnayake, U., Klug, M., Binder, M.D., Hannan, A.J., van den Buuse, M., 2014. Long-term effects of combined neonatal and adolescent stress on brain-derived neurotrophic factor and dopamine receptor expression in the rat forebrain. Biochim. Biophys. Acta - Mol. Basis Dis. 1842, 2126–2135. https://doi.org/10.1016/j.bbadis.2014.08.009

Holly, E.N., Miczek, K.A., 2015. Capturing Individual Differences: Challenges in Animal Models of Posttraumatic Stress Disorder and Drug Abuse. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2015.09.015

Javidi, H., Yadollahie, M., 2012. Post-traumatic stress disorder. Int. J. Occup. Environ. Med.

Kalisch, R., Baker, D.G., Basten, U., Boks, M.P., Bonanno, G.A., Brummelman, E., Chmitorz, A., Fernàndez, G., Fiebach, C.J., Galatzer-Levy, I., Geuze, E., Groppa, S., Helmreich, I., Hendler, T., Hermans, E.J., Jovanovic, T., Kubiak, T., Lieb, K., Lutz, B., Müller, M.B., Murray, R.J., Nievergelt, C.M., Reif, A., Roelofs, K., Rutten, B.P.F., Sander, D., Schick, A., Tüscher, O., Van Diest, I., Van Harmelen, A.L., Veer, I.M., Vermetten, E., Vinkers, C.H., Wager, T.D., Walter, H., Wessa, M., Wibral, M., Kleim, B., 2017. The resilience framework as a strategy to combat stress-related disorders. Nat. Hum. Behav. https://doi.org/10.1038/s41562-017-0200-8

Knox, D., George, S.A., Fitzpatrick, C.J., Rabinak, C.A., Maren, S., Liberzon, I., 2012. Single prolonged stress disrupts retention of extinguished fear in rats. Learn. Mem. https://doi.org/10.1101/lm.024356.111

Kutlu, M.G., Gould, T.J., 2016. Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: Contributions to development and maintenance of addiction. Learn. Mem. https://doi.org/10.1101/lm.042192.116

Lapiz, M.D.S., Fulford, A., Muchimapura, S., Mason, R., Parker, T., Marsden, C.A., 2003. Influence of postweaning social isolation in the rat on brain development, contioned behavior, and neurotransmission. Neurosci. Behav. Physiol. https://doi.org/10.1023/A:1021171129766

Liberzon, I., López, J.F., Flagel, S.B., Vázquez, D.M., Young, E.A., 1999. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: Relevance to post-traumatic stress disorder. J. Neuroendocrinol. 11, 11–17. https://doi.org/10.1046/j.1365-2826.1999.00288.x

Lisieski, M.J., Eagle, A.L., Conti, A.C., Liberzon, I., Perrine, S.A., 2018. Single-prolonged stress:

A review of two decades of progress in a rodent model of post-traumatic stress disorder. Front. Psychiatry. https://doi.org/10.3389/fpsyt.2018.00196

Lissek, S., Grillon, C., 2010. Overgeneralization of conditioned fear in the anxiety disorders putative memorial mechanisms. J. Psychol. https://doi.org/10.1027/0044-3409/a000022

McEwen, B.S., 2007. Physiology and neurobiology of stress and adaptation: Central role of the brain. Physiol. Rev. https://doi.org/10.1152/physrev.00041.2006

McEwen, B.S., 2003. Mood disorders and allostatic load. Biol. Psychiatry. https://doi.org/10.1016/S0006-3223(03)00177-X

McGaugh, J.L., 2013. Making lasting memories: Remembering the significant. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.1301209110

Menesini, E., Salmivalli, C., 2017. Bullying in schools: the state of knowledge and effective interventions. Psychol. Heal. Med. 22, 240–253. https://doi.org/10.1080/13548506.2017.1279740

Müller, K.W., Wölfling, K., 2017. Both sides of the story: Addiction is not a pastime activity: Commentary on: Scholars' open debate paper on the world health organization ICD-11 gaming disorder proposal (Aarseth et al.). J. Behav. Addict. https://doi.org/10.1556/2006.6.2017.038

Mumtaz, F., Khan, M.I., Zubair, M., Dehpour, A.R., 2018. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. Biomed. Pharmacother. https://doi.org/10.1016/j.biopha.2018.05.086

Musazzi, L., Tornese, P., Sala, N., Popoli, M., 2018. What acute stress protocols can tell us about PTSD and stress-related neuropsychiatric disorders. Front. Pharmacol. https://doi.org/10.3389/fphar.2018.00758

Peña, C.J., Kronman, H.G., Walker, D.M., Cates, H.M., Bagot, R.C., Purushothaman, I., Issler, O., Eddie Loh, Y.H., Leong, T., Kiraly, D.D., Goodman, E., Neve, R.L., Shen, L., Nestler, E.J., 2017. Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. Science (80-.). 356, 1185–1188. https://doi.org/10.1126/science.aan4491

Rettew, D.C., Pawlowski, S., 2016. Bullying. Child Adolesc. Psychiatr. Clin. N. Am. https://doi.org/10.1016/j.chc.2015.12.002

Richter-Levin, G., 1998. Acute and long-term behavioral correlates of underwater trauma-potential relevance to stress and post-stress syndromes. Psychiatry Res. 79, 73–83. https://doi.org/10.1016/S0165-1781(98)00030-4

Ritov, G., Boltyansky, B., Richter-Levin, G., 2016. A novel approach to PTSD modeling in rats reveals alternating patterns of limbic activity in different types of stress reaction. Mol. Psychiatry. https://doi.org/10.1038/mp.2015.169

Romeo, R.D., McEwen, B.S., 2006. Stress and the adolescent brain, in: Annals of the New York Academy of Sciences. pp. 202–214. https://doi.org/10.1196/annals.1376.022

Roozendaal, B., McGaugh, J.L., 2011. Memory Modulation. Behav. Neurosci. https://doi.org/10.1037/a0026187

Schacter, D.L., 1999. The seven sins of memory: Insights from psychology and cognitive neuroscience. Am. Psychol. https://doi.org/10.1037/0003-066X.54.3.182

Schönenberg, M., Reichwald, U., Domes, G., Badke, A., Hautzinger, M., 2008. Ketamine aggravates symptoms of acute stress disorder in a naturalistic sample of accident victims. J. Psychopharmacol. 22, 493–497. https://doi.org/10.1177/0269881107082481

Schönenberg, M., Reichwald, U., Domes, G., Badke, A., Hautzinger, M., 2005. Effects of peritraumatic ketamine medication on early and sustained posttraumatic stress symptoms in moderately injured accident victims. Psychopharmacology (Berl). 182, 420–5. https://doi.org/10.1007/s00213-005-0094-4

Sillivan, S.E., Joseph, N.F., Jamieson, S., King, M.L., Chévere-Torres, I., Fuentes, I., Shumyatsky, G.P., Brantley, A.F., Rumbaugh, G., Miller, C.A., 2017. Susceptibility and Resilience to Posttraumatic Stress Disorder–like Behaviors in Inbred Mice. Biol. Psychiatry 82, 924–933. https://doi.org/10.1016/j.biopsych.2017.06.030

Skelton, K., Ressler, K.J., Norrholm, S.D., Jovanovic, T., Bradley-Davino, B., 2012. PTSD and gene variants: New pathways and new thinking. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2011.02.013

Souza, R.R., Noble, L.J., McIntyre, C.K., 2017. Using the single prolonged stress model to examine the pathophysiology of PTSD. Front. Pharmacol. 8. https://doi.org/10.3389/fphar.2017.00615

Spear, L.P., 2000a. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev. https://doi.org/10.1016/S0149-7634(00)00014-2

Tsoory, M., Cohen, H., Richter-Levin, G., 2007. Juvenile stress induces a predisposition to either anxiety or depressive-like symptoms following stress in adulthood. Eur. Neuropsychopharmacol. 17, 245–256. https://doi.org/10.1016/j.euroneuro.2006.06.007

Ullmann, E., Perry, S.W., Licinio, J., Wong, M.L., Dremencov, E., Zavjalov, E.L., Shevelev, O.B., Khotskin, N. V., Koncevaya, G. V., Khotshkina, A.S., Moshkin, M.P., Lapshin, M.S., Komelkova, M. V., Feklicheva, I. V., Tseilikman, O.B., Cherkasova, O.P., Bhui, K.S., Jones, E., Kirschbaum, C., Bornstein, S.R., Tseilikman, V., 2019. From allostatic load to allostatic state—an endogenous sympathetic strategy to deal with chronic anxiety and stress? Front. Behav. Neurosci. https://doi.org/10.3389/fnbeh.2019.00047

Verbitsky, A., Dopfel, D., Zhang, N., 2020. Rodent models of post-traumatic stress disorder: behavioral assessment. Transl. Psychiatry. https://doi.org/10.1038/s41398-020-0806-x

Von Der Heiden, J.M., Braun, B., Müller, K.W., Egloff, B., 2019. The association between video gaming and psychological functioning. Front. Psychol. https://doi.org/10.3389/fpsyg.2019.01731

Winter, H., Irle, E., 2004. Hippocampal volume in adult burn patients with and without

posttraumatic stress disorder. Am. J. Psychiatry 161, 2194–200. https://doi.org/10.1176/appi.ajp.161.12.2194

Wu, G., Feder, A., Cohen, H., Kim, J.J., Calderon, S., Charney, D.S., Mathé, A.A., 2013. Understanding resilience. Front. Behav. Neurosci. https://doi.org/10.3389/fnbeh.2013.00010

Wu, Z., Tian, Q., Li, F., Gao, J., Liu, Y., Mao, M., Liu, J., Wang, S., Li, G., Ge, D., Mao, Y., Zhang, W., Liu, Z., Song, Y., 2016. Behavioral changes over time in post-traumatic stress disorder: Insights from a rat model of single prolonged stress. Behav. Processes. https://doi.org/10.1016/j.beproc.2016.01.001

Yamamoto, S., Morinobu, S., Takei, S., Fuchikami, M., Matsuki, A., Yamawaki, S., Liberzon, I., 2009. Single prolonged stress: Toward an animal model of posttraumatic stress disorder. Depress. Anxiety. https://doi.org/10.1002/da.20629

Yehuda, R., Hoge, C.W., McFarlane, A.C., Vermetten, E., Lanius, R.A., Nievergelt, C.M., Hobfoll, S.E., Koenen, K.C., Neylan, T.C., Hyman, S.E., 2015. Post-traumatic stress disorder. Nat. Rev. Dis. Prim. https://doi.org/10.1038/nrdp.2015.57

Zoladz, P.R., Diamond, D.M., 2016. Predator-based psychosocial stress animal model of PTSD: Preclinical assessment of traumatic stress at cognitive, hormonal, pharmacological, cardiovascular and epigenetic levels of analysis. Exp. Neurol. https://doi.org/10.1016/j.expneurol.2016.06.003

CHAPTER 1

AMPHETAMINE AND THE SMART DRUG 3,4-METHYLENEDIOXYPYROVALERONE (MDPV) INDUCE GENERALIZATION OF FEAR MEMORY IN RATS

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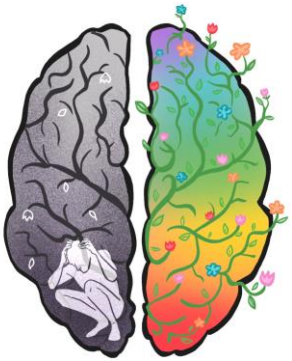

Abstract

Human studies have consistently shown that drugs of abuse affect memory function. The psychostimulants amphetamine and the "bath salt" 3,4-methylenedioxypyrovalerone (MDPV) increase brain monoamine levels through a similar, yet not identical, mechanism of action. Findings indicate that amphetamine enhances the consolidation of memory for emotional experiences, but still MDPV effects on memory function are underinvestigated. Here, we tested the effects induced by these two drugs on generalization of fear memory and their relative neurobiological underpinnings. To this aim, we used a modified version of the classical inhibitory avoidance task, termed *inhibitory avoidance discrimination* task. According to such procedure, adult male Sprague-Dawley rats were first exposed to one inhibitory avoidance apparatus and, with a 1-min delay, to a second apparatus where they received an inescapable footshock. Forty-eight hours later, retention latencies were tested, in a randomized order, in the two training apparatuses as well as in a novel contextually modified apparatus to assess both strength and generalization of memory. Our results indicated that both amphetamine and MDPV induced generalization of fear memory, whereas only amphetamine enhanced memory strength. Co-administration of the β- adrenoceptor antagonist propranolol prevented the effects of both amphetamine and MDPV on strength and generalization of memory. The dopaminergic receptor blocker cis-flupenthixol selectively reversed the amphetamine effect on memory generalization. These findings indicate that amphetamine and MDPV induce generalization of fear memory through different modulations of noradrenergic and dopaminergic neurotransmission.

Introduction

Drugs of abuse are characterized by rewarding effects induced by the engagement of specific pathways in the brain (McHugh and Kneeland, 2019). Such rewarding effects are the principal reason that moves people to a compulsive use of these substances, which frequently ends with drug dependence (Koob, 2017). It has long been observed in humans that the intake of drugs of abuse affects memory processes (Kutlu and Gould, 2016; Goodman and Packard, 2016). More specific studies conducted in laboratory animals have been focused on which neurobiological and biochemical pathways are exploited by drugs of abuse to influence memory. Amphetamine, one of the most well-known psychostimulants, has been shown to enhance the consolidation of memory processing in rodents (McGaugh, 1973; Martinez et al., 1980a; Martinez et al., 1980b; Roozendaal et al., 1996; McGaugh and Roozendaal, 2009). We recently demonstrated that the 3,4-methylenedioxypyrovalerone (MDPV), a newer synthetic cathinone also known as "bath salt", enhances short-term spatial and recognition memory performance (Atehortua-Martinez et al., 2019). Moreover, it has been shown that MDPV induces a disruption of functional connectivity networks (i.e., striatum) involved in cognitive processes (Colon-Perez et al., 2016). This new psychostimulant has recently emerged in the illegal market as a smart drug and it rapidly became highly popular (Prosser and Nelson, 2012; Baumann et al., 2017). However, its fame is also associated with several important adverse effects, and among these, long-term cognitive impairments in humans have been documented (Karila et al., 2015). One *in vitro* study on MDPV activity demonstrated that it has a similar, yet not identical, mechanism of action compared to amphetamine. Indeed, both drugs of abuse have the same molecular targets represented by the norepinephrine (NE) , dopamine (DA) and serotonin re-uptake transporters (NET, DAT and SERT, respectively), but MDPV displays greater potency than amphetamine with regard to DA re-uptake transport (Baumann et al., 2013). Amphetamine effects on memory consolidation

are dependent on its pharmacological action which increases NE and DA release (Martinez et al., 1983; Fleckenstein et al., 2007; LaLumiere et al., 2005; Roozendaal et al., 2008). Very recently, it has been shown that the effect on short-term memory induced by MDPV is linked to D1 dopaminergic receptor activation (Atehortua-Martinez et al., 2019). The role of noradrenergic and dopaminergic neurotransmission on memory, especially for the consolidation phase, is well established (LaLumiere et al., 2005; Roozendaal et al., 2008; Wideman et al., 2018; Quaedflieg and Schwabe, 2018; Schwabe, 2017). Although it has been demonstrated that both amphetamine and MDPV can affect memory retention, no evidence exists on whether such drugs can also affect the quality of memory. The study about the influence of drugs of abuse on the quality of memory increasingly acquired attention during last century and is just nowadays growingly becoming an intriguing issue, even if up to date there are only sparse studies (Koriat et al., 2000; Oeberst and Blank, 2012; Hoscheidt et al., 2014; Loftus, 2005, Horry et al., 2014; Carter et al., 2013; Easton and Bauer, 1997; Ballard et al., 2012). However, the study of the mechanisms through which drugs of abuse affect memory quality could be a riveting topic, mainly in the light of increasing evidence that drugs of abuse (e.g. psychedelic drugs, hallucinogens) can alter the experience of reality (Bohling, 2017). Such altered perception might be one of the causes why some people are prompted to a recreational use of such substances (Moro et al., 2011; Kjellgren and Soussan, 2011), thus making it an important and urgent issue to be investigated. Emotions have a considerable impact on memory (Tyng et al., 2017), for example, when an aversive stimulus occurs, the associated fear leads to remembering the information over time (Rogan et al., 1997), but sometimes the accuracy of such emotional memory can be altered and distorted over time, eventually leading to memory generalization (Asok et al., 2018). This emotional/fear generalization effect has been studied for many decades through the contextual fear conditioning paradigm (Rohrbaugh and Riccio, 1968; Ruediger et al., 2011). Recently, a novel experimental model suitable to

investigate both strength and accuracy of memory has been validated for rodents (Atucha and Roozendaal, 2015, Atucha et al., 2017): the inhibitory avoidance discrimination task. This task allows to evaluate whether fear memory associated with footshock can be generalized to a novel and safe, yet similar. context. Hence, the aim of the present study was to investigate whether the two psychostimulants amphetamine and MDPV affect generalization of fear memory to a novel and safe yet similar context using an inhibitory avoidance discrimination task. Since both amphetamine and MDPV modulate NE and DA tone, we also aimed at evaluating the involvement of the noradrenergic and dopaminergic systems in mediating the effects of amphetamine and MDPV on fear memory generalization.

Materials and Methods

Animals and procedures

Male adult Sprague-Dawley rats (320–370 g at the time of behavioral experiments) from Charles River Laboratories (Calco, Italy) were housed individually in a temperature-controlled (21 ± 1°C) vivarium room and maintained under a 12 h/12 h light/dark cycle (7:00 A.M. to 7:00 P.M. lights on). Food and water were available *ad libitum*. Rats were handled for 1 min for 3 consecutive days prior to training. Training and testing were performed during the light phase of the cycle between 11:00 A.M. and 2:00 P.M. All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, Directive 2010/63/EU of the European Parliament, the D. L. 26/2014 of the Italian Ministry of Health, the Declaration of Helsinki and the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2004).

Inhibitory avoidance discrimination task

For all experiments, rats were trained and tested on a modified version of the classic inhibitory avoidance task, termed inhibitory avoidance discrimination task, that

allows to investigate strength and accuracy of memory (Atucha and Roozendaal, 2015, Atucha et al., 2017). Rats were subsequently trained in two contextually distinct inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in the latter context. On the retention test, they were tested in both training contexts as well as in a novel context. These training and test procedures, as previously demonstrated by Atucha and Roozendaal (2015), allow to investigate whether rats remember the two contexts they visited during the training trial, as well as if they display a specific episodic-like memory of the association between footshock and the correct training context. Each apparatus had the same geometry and consisted of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor. The starting compartment (31 cm) was made of opaque white plastic and was well lit; the shock compartment (60 cm) was made of two dark, electrifiable metal plates and was not illuminated. The training context in which footshock was given (Shock box) did not have any contextual modifications. The safe training context (Non-Shock box) had four vertical white stripes (2 cm wide) taped in the dark compartment together with tape placed on the floor, closing the gap between the two plates. The Novel box (used on the retention test only) had two white circles (3.5 cm diameter) taped on each wall of the dark compartment, and the gap between the plates was closed with tape. All three inhibitory avoidance apparatuses were located next to one another in a sound- and light-attenuated room.

For training, rats were initially placed in the starting compartment of the Non-Shock box and their latency to enter the dark compartment with all four paws (maximum latency of 30 s) was recorded. No footshock was delivered in this box. Afterward, the rats were removed from the apparatus and, after a delay of 1-min, placed in the starting compartment of the second inhibitory avoidance apparatus (Shock box). We selected a 1-min delay because, as previously demonstrated (Atucha and Roozendaal, 2015), although animals do not discriminate between the two training contexts with such short interval between the two training episodes, the fear does not generalize to a novel

context. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.30 mA; 1 s) was delivered. The rats were removed from the apparatus 20 s after termination of footshock and, after drug treatment, returned to their home cages. On the retention test, two days after training, they were tested, in a randomized order and without delay, in the two training contexts (i.e., Shock box and Non-Shock box) and in a Novel box they had not visited before. No footshock was delivered on the retention test trial, and for all three boxes, the rats were placed in the starting compartment and their latency to enter the dark compartment with all four paws (maximum latency of 600 s) was recorded. Longer latencies in the Shock box compared with the Non-Shock or Novel box were interpreted as indicating accurate memory of the shock–context association. Moreover, long retention latencies in all the three boxes were considered as an index of memory generalization across contexts. Immediately after the training or testing of each animal, each apparatus was wiped clean with a 70% ethanol solution. The experimental design is illustrated in Fig. 1.

Drug administration

Amphetamine ((RS)-1-phenylpropan-2-amine) (1 and 3 mg/kg) and MDPV (3,4-methylenedioxypyrovalerone) (0.5 and 1 mg/kg) were dissolved in saline (vehicle) and administered intraperitoneally, at the volume of 1 ml/kg, immediately after the training session (Fig. 1). In the second experiment, to examine whether the amphetamine and MDPV effects on memory involve the noradrenergic system, the β-adrenoceptor antagonist propranolol (1-naphthalen-1-yloxy-3-propan-2-ylaminopropan-2-ol) (1 mg/kg) or saline (vehicle) was administered intraperitoneally 30 min prior to training, followed by amphetamine (3 mg/kg), MDPV (1 mg/kg) or saline immediately after training (Fig. 1). In the third experiment, to investigate the involvement of the dopaminergic system in mediating amphetamine and MDPV effects on memory, the non-selective D1/D2 dopaminergic receptor antagonist cis-flupenthixol (2-[4-[(3Z)-3-[2-(trifluoromethyl)thioxanthen- 9-

ylidene]propyl]piperazin-1-yl]ethanol) (0.25 mg/kg) or saline (vehicle) was administered intraperitoneally 30 min prior to training, followed by an immediate post-training intraperitoneal injection of amphetamine (3 mg/kg), MDPV (1 mg/kg) or saline (Fig. 1). Drug doses were chosen on the basis of literature data (Roozendaal et al., 2004, Trost and Hauber, 2014) also showing that MPDV has a greater pharmacological potency than amphetamine (Bauman et al, 2013). All drugs were dissolved in sterile 0.9% saline. Drug solutions were freshly prepared before each experiment.

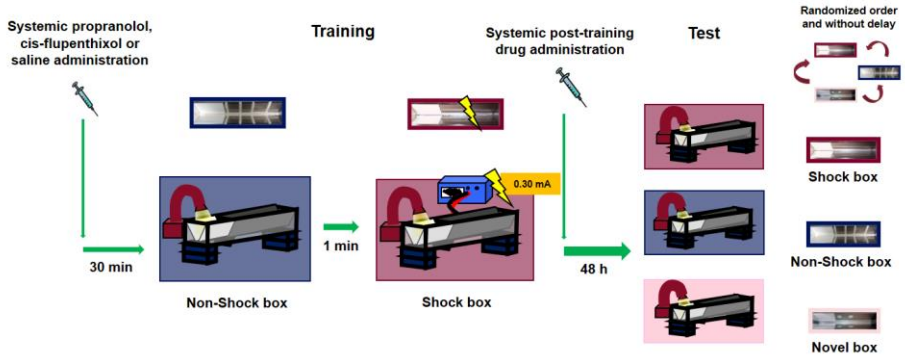

Figure 1: Schematic representation of the experimental design.

Statistical analysis

Data are expressed as mean ± SEM. All data were analyzed with ANOVA for Repeated Measures (RM ANOVA) with drug treatment as between-group factor and retention latencies of individual animals in the different test contexts (Shock, Non-Shock, and Novel boxes) as repeated measure. Two-way ANOVAs were used to analyze retention latencies of rats treated with propranolol vs saline alone and cis-flupenthixol vs saline alone. The source of the detected significances was determined by Tukey–Kramer *post hoc* tests for between and within-group differences. P values of less than 0.05 were considered statistically significant. The number of rats per group is indicated in the figure legends.

Results

Amphetamine and MDPV induce memory generalization in an inhibitory avoidance discrimination task

Rats were trained on the inhibitory avoidance discrimination task and given an immediate post-training intraperitoneal injection of amphetamine, MDPV or saline. With regard to amphetamine effects, as shown in Fig. 2a, RM ANOVA for retention latencies indicated significant effects for treatment ($F_{(2,29)} = 10.23$, $P < 0.01$) as well as context ($F_{(2,29)} = 4.08$, $P = 0.02$), but no significant interaction between these two factors ($F_{(4,58)} = 0.48$, $P = 0.75$). *Post-hoc* analysis, in accordance to what it has been previously demonstrated (Atucha and Roozendaal, 2015), revealed that saline- treated animals showed longer retention latencies in the Shock box ($P < 0.01$) and Non-Shock box ($P < 0.01$) compared to those in the Novel box, indicating that saline- treated rats were able to discriminate the two training contexts from the new one they had visited only during the test trial (Fig. 2a). Retention latencies in the Shock box of rats treated with amphetamine (3 mg/kg) were significantly longer than those of animals treated with saline ($P < 0.05$), indicating that amphetamine, at the higher dose tested, enhanced the strength of memory. Furthermore, amphetamine (3 mg/kg)-treated rats showed longer retention latencies in both the Non-Shock box ($P < 0.05$) and Novel box ($P < 0.01$) compared to saline-treated animals. Thus, these results revealed that amphetamine induced memory generalization across contexts. With regard to MDPV effects, as shown in Fig. 2b, RM ANOVA for retention latencies indicated no significant effect for treatment ($F_{(2,30)} = 1.83$, $P = 0.18$), a significant context effect ($F_{(2,30)} = 3.37$, $P = 0.04$), and no significant interaction between these two factors ($F_{(2,60)} = 1.04$, $P = 0.39$). *Post-hoc* analysis confirmed that the performance of control animals was the same as for the amphetamine experiments (Fig. 2b). Retention latencies of animals treated with MDPV (1 mg/kg) did not differ from those of saline-treated controls in both Shock and Non-Shock boxes, but were significantly longer than those of saline-treated animals ($P < 0.05$) in the Novel box. These results show

that rats that were treated with MDPV (1 mg/kg) had similar retention latencies in all three boxes, indicating that MDPV induced generalization across contexts. Taken together, these findings indicate that amphetamine and MDPV have differential effects on memory strength, but that both drugs increase generalization of fear memory to a novel safe context. All training latencies are shown in Supplementary Table 1.

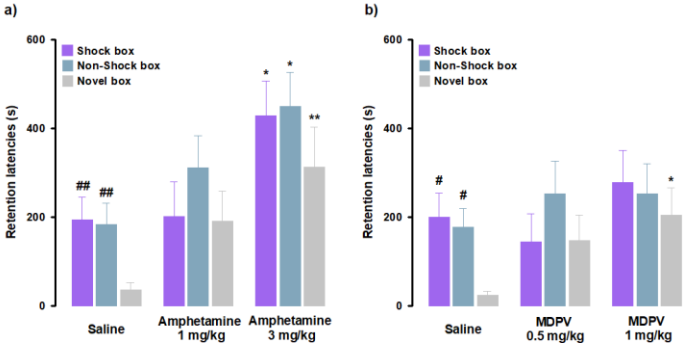

Figure 2: Amphetamine and MDPV induce memory generalization of inhibitory avoidance discrimination task. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. a) Retention latencies of amphetamine and saline-treated rats. Saline-treated animals showed longer retention latencies in the Shock box and Non- Shock box compared to those induced in the Novel box. In all three boxes, amphetamine 3 mg/kg induced higher retention latencies than saline-treated rats. ##, $P < 0.01$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; *, $P< 0.05$, **, $P < 0.01$ amphetamine 3 mg/kg latencies in the Shock box, Non-Shock box or Novel box vs saline group in the Shock box, Non-Shock box or Novel box; NS, no significant differences (n = 9-13 rats). b) Retention latencies of MDPV and saline-treated rats. Saline- treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box. In the Novel box retention latencies induced by MDPV 1 mg/kg were significantly longer than those induced by saline-treated rats in the same box. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; *, $P < 0.05$ MDPV 1 mg/kg treated group latencies in the Novel box vs saline group latencies in the Novel box; NS, no significant differences (n = 10-12 rats).

Noradrenergic system activation mediates the effects of amphetamine and MDPV on memory generalization

We sought to test whether the amphetamine- and MDPV-mediated effects on strength and generalization of memory involved activation of the noradrenergic system. Here in, rats were given intraperitoneal injections of the β-adrenoceptor antagonist propranolol or saline 30 min prior to training, followed by post-training administrations of the effective doses of amphetamine (3 mg/kg), MDPV (1 mg/kg), or their corresponding vehicles. To investigate whether the noradrenergic system influences on amphetamine- mediated effects on memory generalization, we first analyzed retention latencies of saline- and propranolol alone-treated animals in the three contexts (Fig. 3a). RM ANOVA for retention latencies of the saline-treated animals showed a significant effect of context ($F_{(2,36)} = 4.80$, $P = 0.01$). Similar to the control rats described above, *post-hoc* analysis confirmed that saline-treated animals showed longer retention latencies in the Shock box ($P < 0.05$) and Non-Shock box ($P < 0.05$) as compared to those in the Novel box, thus indicating that control rats were able to discriminate the two training contexts from the new one that they visited only during the test trial. The same results were obtained with the RM ANOVA analysis for retention latencies of propranolol alone-treated animals ($F_{(2,35)} = 4.52$, $P = 0.02$). *Post-hoc* analysis revealed that propranolol alone-treated rats showed longer retention latencies in the Shock box ($P < 0.05$) and Non-Shock box ($P < 0.05$) as compared to those in the Novel box. These findings indicate that also rats that were treated with propranolol accurately remembered the two training contexts, even if they were not able to discriminate in which training context they received the footshock. Moreover, two-way ANOVA for retention latencies of rats treated with saline and propranolol did not reveal a significant treatment effect ($F_{(1,69)} = 0.59$, $P = 0.44$) or treatment x context interaction effect ($F_{(2,69)} = 0.03$, $P = 0.97$), but revealed a significant effect of the context ($F_{(2,69)} = 9.23$, $P < 0.0001$), suggesting that treatment does not affect animals memory retention for different apparatuses (Fig. 3a). As shown in Fig. 3a, as

for the noradrenergic influences in the amphetamine effects on memory function, RM-ANOVA for retention latencies revealed significant effects of treatment ($F_{(3,42)}$ = 11.70, $P < 0.01$) as well as context ($F_{(2,42)}$ = 6.01, $P < 0.01$), and no significant differences for the interaction between both factors ($F_{(6,84)}$ = 0.50, P = 0.80). Retention latencies of rats treated with amphetamine alone in the Shock box ($P < 0.05$), Non-Shock box ($P < 0.05$) and Novel box ($P < 0.01$) were all significantly longer than those displayed by saline-treated animals in the same boxes. Retention latencies of rats that were treated with propranolol together with amphetamine in the Shock box ($P < 0.05$), Non-Shock box ($P < 0.01$) and Novel box ($P < 0.01$) were significantly shorter compared to those of animals treated with amphetamine alone in the same boxes. Moreover, retention latencies of rats treated with amphetamine alone in the Shock box ($P < 0.05$), Non-Shock box ($P < 0.01$) and Novel box ($P < 0.01$) were significantly longer than those of rats treated with propranolol alone in the same boxes. To evaluate whether noradrenergic activity is also involved in the modulation of the MDPV effects on memory generalization, we analyzed retention latencies of both saline and propranolol alone-treated animals and confirmed the results that we described above for the experiments involving amphetamine (Fig. 3b). Furthermore, as previously described, also in this experiment no significant differences were found between saline and propranolol alone-treated rats (Fig. 3b).

As shown in Fig. 3b, RM ANOVA for retention latencies indicated no significant effect of treatment ($F_{(3,32)}$ = 1.70, P = 0.19) or treatment x context interaction effect ($F_{(6,64)}$ = 1.12, P = 0.36), but revealed a significant effect of the context ($F_{(2,32)}$ = 7.32, $P < 0.01$). Rats treated with MDPV alone showed longer retention latencies in the Novel box than those of saline alone- ($P < 0.01$) or propranolol alone-treated rats ($P < 0.05$) exposed to the same box. Moreover, retention latencies of animals treated with propranolol together with MDPV in the Shock-box were significantly longer compared to the Novel box ($P < 0.05$) and in the Non-Shock box compared to the Novel box ($P < 0.05$). Particularly in the Novel box, retention latencies of animals

treated with propranolol together with MDPV were significantly shorter compared to those of MDPV alone-treated animals in the same box.In summary, these findings indicate that the amphetamine effect on enhancing memory strength is mediated by the noradrenergic system. Moreover, our findings indicate that the amphetamine effect on memory generalization appears to be only partially due to a modulation of the noradrenergic system, whereas the memory generalization effect induced by MDPV is entirely dependent on noradrenergic activity. All training latencies are indicated in Supplementary Table 2

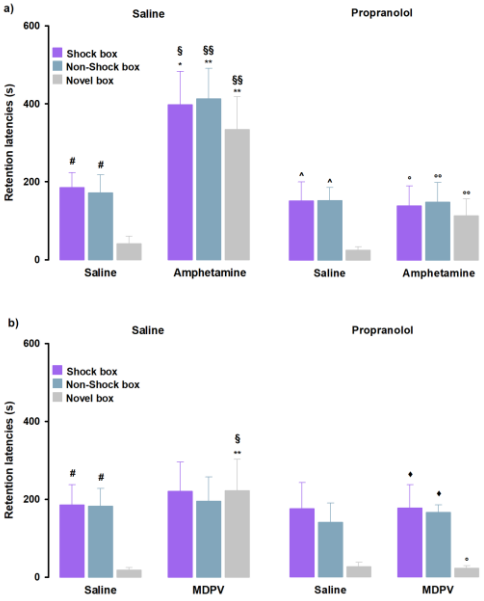

Figure 3: Noradrenergic activation mediates amphetamine and MDPV effects on memory generalization. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. a) Retention latencies of rats treated with propranolol or saline 30 min prior to training together with amphetamine or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box, the same happens for the propranolol alone-treated

animals. In all three boxes, amphetamine alone-treated rats showed higher retention latencies than saline alone-treated rats and then those exerted by rats given propranolol alone. Retention latencies of group treated with propranolol together with amphetamine in all three boxes were significantly lower compared to those of amphetamine alone-treated rats. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ^, $P < 0.05$ propranolol alone latencies in the Shock box or Non-Shock box vs propranolol alone latencies in the Novel box; *, $P < 0.05$, **, P < amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box vs saline group latencies in the Shock box, Non-Shock box or Novel box; §, $P < 0.05$, §§, $P < 0.01$ amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box vs propranolol alone group latencies in the Shock box, Non-Shock box or Novel box; °, $P < 0.05$, °°, $P < 0.01$ propranolol and amphetamine-treated group latencies in the Shock box, Non-Shock box or Novel box vs amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box; NS, no significant differences (n = 9-13 rats). b) Retention latencies of rats treated with propranolol or saline 30 min prior to training together with MDPV or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box, the same happens for the propranolol together with MDPV-treated animals. In the Novel box retention latencies induced by MDPV alone treatment were significantly longer than those exerted by rats treated with saline alone and propranolol alone. Retention latencies of group treated with propranolol together with MDPV in the Novel box were significantly lower compared to those of MDPV alone-treated rats. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ♦, $P < 0.05$ propranolol together with MDPV latencies in the Shock box or Non-Shock box vs propranolol together with MDPV latencies in the Novel box; **, $P < 0.01$, MDPV alone-treated group latencies in the Novel box vs saline group latencies in the Novel box; §, $P < 0.05$, MDPV alone-treated group latencies in the Novel box vs propranolol alone-treated group latencies in the Novel box; °, $P < 0.05$, propranolol and MDPV-treated group latencies in the Novel box vs MDPV alone-treated group in the Novel box; NS, no significant differences (n = 8-11 rats).

Dopaminergic system activation mediates the effects of amphetamine, but not MDPV, on memory generalization

In this set of experiments, we tested whether dopaminergic activity is involved in the effects induced by amphetamine and MDPV on memory generalization. To this aim, rats were intraperitoneally treated with the dopamine receptors antagonist cis-flupenthixol or saline 30 min before the training trial, and subjected to post-training administration of the effective doses of amphetamine (3 mg/kg), MDPV (1 mg/kg), or their corresponding vehicle solutions. As previously done in the experiments involving the noradrenergic system, we first analyzed the retention latencies of saline- and of cis-flupenthixol alone-treated animals in the three experimental contexts. Animals that were treated with saline showed comparable latencies to control groups that were discussed above (Fig. 4a). Moreover, in line with the previous set of experiments, no significant differences between saline- and cis-flupenthixol alone-treated animals (Fig. 4a) were detected. As for the involvement of the dopaminergic system in the amphetamine effects on memory function, as shown in Fig. 4a, RM ANOVA for retention latencies indicated significant effects of treatment ($F_{(3,34)} = 10.87$, $P < 0.01$) and context ($F_{(2,34)} = 17.62$, $P < 0.01$), but not significant interaction between both factors ($F_{(6,68)} = 0.47$, $P = 0.83$) effect. *Post-hoc* analysis revealed that retention latencies of amphetamine alone- treated rats were significantly longer than those of rats that were given saline alone in the Shock box ($P < 0.05$), Non-Shock box ($P < 0.05$) and Novel box ($P < 0.01$). Retention latencies of rats that were treated with amphetamine alone were significantly longer than those of cis-flupenthixol alone-treated rats in the Shock box ($P < 0.05$), Non-Shock box ($P < 0.01$) and Novel box ($P < 0.01$). Retention latencies in the Novel box of rats treated with cis-flupenthixol together with amphetamine were significantly shorter with respect to rats given amphetamine alone ($P < 0.01$) in the same box. Moreover, they showed longer latencies in the Shock box and in the Non-Shock box compared to the Novel box ($P < 0.05$).

Concerning the dopaminergic role on MDPV-mediated generalization effects on memory, for the retention latencies of both saline- and cis-flupenthixol alone-treated rats we confirmed the same results of above described (Fig. 4b); again, no significant differences were found between the two treatment groups (Fig. 4b). As shown in Fig. 4b, RM ANOVA for retention latencies indicated no significant treatment effect ($F_{(3,38)}$ = 1.71, P = 0.18), a significant effect of the context ($F_{(2,38)}$ = 5.06, P < 0.01) and no significant interaction between these two factors ($F_{(6,76)}$ = 0.81, P = 0.56) effect. *Post-hoc* analysis revealed that retention latencies of rats treated with MDPV alone were significantly longer than those of rats given saline alone and cis- flupenthixol alone in the Novel box (P < 0.01), and that the retention latencies of rats treated with cis-flupenthixol together with MDPV were significantly longer than those of rats given saline alone and cis-flupenthixol alone in the Novel box (P < 0.05).

In conclusion, these results demonstrated that the dopaminergic system is involved in modulating the effects of amphetamine on memory generalization as well with only a partial interference on its effects on memory strength. However, the blockade of dopamine receptors does not influence MDPV effects on memory generalization. All training latencies are shown in Supplementary Table 3.

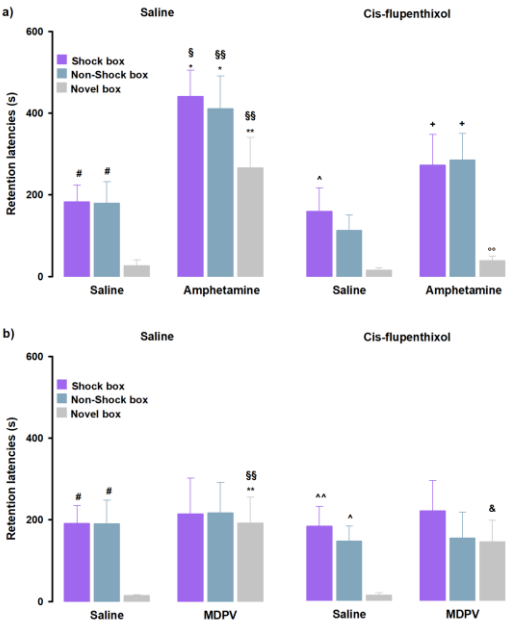

Figure 4: Dopaminergic activation mediates the effects induced by amphetamine, but not MDPV, on memory generalization. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. a) Retention latencies of rats treated with cis- flupenthixol or saline 30 min prior to training together with amphetamine or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box. Cis-flupenthixol alone-treated animals showed higher retention latencies in Shock box compared only to those showed in the Novel box. Cis-flupenthixol together with amphetamine treated-rats showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box. In all three boxes, amphetamine alone-treated rats showed higher retention latencies than saline alone-treated rats and cis-flupenthixol alone-treated rats. Retention latencies of rats treated with cis-flupenthixol together with amphetamine were significantly lower than those of amphetamine alone-treated rats, only in the Novel box. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ^, $P < 0.05$ cis-flupenthixol alone

latencies in the Shock box vs cis-flupenthixol alone latencies in the Novel box; +, $P < 0.05$, cis-flupenthixol together with amphetamine latencies in the Shock or Non-Shock box vs cis-flupenthixol together with amphetamine latencies in the Novel box; *, $P < 0.05$, ** $P < 0.01$, amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box vs saline group latencies in the Shock box, Non-Shock box or Novel box; §, $P < 0.05$, §§, $P < 0.01$, amphetamine alone group latencies in the Shock box, Non-Shock box or Novel box vs cis-flupenthixol alone-treated group latencies in the Shock box, Non-Shock box or Novel box; °°, $P < 0.01$, cis-flupenthixol and amphetamine-treated group latencies in the Novel box vs amphetamine alone-treated group in the Novel box; NS, no significant differences (n= 9-10 rats). b) Retention latencies of rats treated with cis-flupenthixol or saline 30 min prior to training together with MDPV or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box, the same happens to cis-flupenthixol alone-treated animals. In the Novel box, MDPV alone- treated rats showed higher latencies with respect to saline-treated rats and cis-flupenthixol alone-treated rats; cis-flupenthixol and MDPV-treated rats showed higher latencies with respect to cis-flupenthixol alone-treated rats and with respect to cis-flupenthixol alone-treated. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ^, $P < 0.05$, ^^, $P < 0.01$, cis-flupenthixol alone latencies in the Shock box or Non-shock box vs cis-flupenthixol alone latencies in the Novel box; **, $P < 0.01$, MDPV alone-treated group latencies in the Novel box vs saline group latencies in the Novel box; §§, $P < 0.01$, MDPV alone-treated group latencies in the Novel box vs cis-flupenthixol alone- treated group in the Novel box; &, $P < 0.05$, cis-flupenthixol together with MDPV retention latencies in the Novel box vs cis-flupenthixol alone latencies in the Novel box; NS, no significant differences (n = 8-11 rats).

Discussion

The present findings indicate that amphetamine and MDPV have different effects on memory strength, but both drugs increase generalization of fear memory to a novel safe context. We further show that noradrenergic and dopaminergic neurotransmission is differentially involved in the effects mediated by amphetamine and MDPV on memory. As previously showed, saline-treated animals trained in the inhibitory

avoidance discrimination task, with a 1-min interval between the two training apparatuses, were able to discriminate the two training contexts from the new one visited only during the test trial (Atucha and Roozendaal, 2015), indicating that fear memory associated with footshock did not generalize to the novel safe box. Here, we specifically selected this short time delay to evaluate whether amphetamine and MDPV could induce fear memory generalization of footshock to the novel safe context. Our findings first demonstrate, in accordance to previous reports (McGaugh, 1973; Martinez et al., 1980a; Martinez et al., 1980b; Roozendaal et al., 1996, McGaugh and Roozendaal, 2009), that amphetamine increases memory strength as indicated by the longer retention latencies in the Shock box. Of more interest, we also found that amphetamine induces fear memory generalization by enhancing retention latencies in all three boxes, including the box never visited before. MDPV did not directly affect memory strength, but induced generalization of memory, as well as demonstrated by the finding that MDPV-treated animals exerted similar retention latencies in all three boxes. Such evidence that both psychostimulants induce fear memory generalization to a context to which animals were never exposed before is a truly novel and important finding.

Previous studies have indicated that both amphetamine and MDPV, through a similar, yet not identical, mechanism of action increase brain monoamines release, particularly NE and DA, two neurotransmitters extensively involved in the modulation of memory (LaLumiere et al., 2005; McGaugh and Roozendaal, 2009). In fact, amphetamine acts as a substrate of NET, DAT and SERT inducing a 'reverse transport' of neurotransmitters (Robertson et al., 2009), whereas MDPV, like cocaine, is an inhibitor of NET, DAT and SERT (Simmler et al., 2013; Marusich et al., 2014; Baumann et al., 2017). Amphetamine also interacts with the vesicular monoamine transporter (VMAT), in particular VMAT2, depleting synaptic vesicles of their neurotransmitter content (Teng et al., 1998; Eiden and Weihe, 2011), and inhibits monoaminooxidase (MAO), which is a family of enzymes that catalyzes monoamine

oxidation (Miller et al., 1980; Liu et al., 2016). The affinity between MDPV and MAO has not yet been investigated. Literature data indicate that two other synthetic cathinones, mephedrone and methylone, have a similar mechanism of action of amphetamine but present a lower affinity for VMAT2 and probably decrease activity on MAO with respect to amphetamine (Baumann et al., 2017). There is evidence that MDPV is more powerful as an uptake blocker of DAT than of NET and SERT (Baumann et al., 2007). Therefore, although this remains purely speculative, it is possible that the different effects induced by amphetamine and MDPV on memory strength may be related to variation of the specific expressions of these monoamine transporters in different brain regions.

Notwithstanding the different mechanism of action through which these two psychostimulants enhance NE and DA levels, both drugs of abuse enhance noradrenergic and dopaminergic neurotransmission (Baumann et al., 2013; Robertson et al., 2009) and the involvement of these two systems on the effects induced by drugs of abuse on memory strength and generalization had not been previously investigated. Here, we found that noradrenergic influences, mediated by an action on β-adrenoceptors, were responsible for the enhancing effects of amphetamine on memory consolidation. Extensive evidence indicates that noradrenergic activation is crucially involved in regulating memory consolidation for emotional experiences (Gold et al., 1975; Gold and van Buskirk, 1978; Gallagher et al., 1977; Liang et al., 1986; McIntyre et al., 2003; Ferry et al., 2015; LaLumiere et al., 2017). Hence, it is possible that amphetamine effects on memory strength could be due to an indirect activation of central β-adrenoceptors. Of more novel interest, we demonstrated that the noradrenergic system also modulates the generalization effects induced by both amphetamine and MDPV. In particular, our findings indicate that amphetamine effects on generalization are partially blocked by preventive administration of the β-adrenoceptor antagonist propranolol, while MDPV effects are totally blocked. Previous findings demonstrated that the administration of the physiological

noradrenergic stimulant yohimbine, a selective α2-adrenoceptor antagonist, ameliorates the accuracy of memory in the inhibitory avoidance discrimination task (Atucha and Roozendaal, 2015) and that NA infusion into the basolateral amygdala maintains accuracy of episodic-like memory of the two distinct training contexts, preventing the generalization effect induced by a memory reorganization over time (Atucha et al., 2017). However, our results unexpectedly suggest that if the noradrenergic system is activated by a drug of abuse it alters memory accuracy, inducing generalization. This effect could be explained considering the activation of the noradrenergic system in brain areas particularly involved in memory generalization, such as medial prefrontal cortex, nucleus reunions, and hippocampus (Xu and Sudhof, 2013). Conversely, no data are available with regard to the potential role of dopaminergic modulation on memory accuracy. Herein, we demonstrate that the dopaminergic system is involved in modulating the effects of amphetamine on memory generalization as well with only a partial interference on memory strength. However, the blockade of dopamine receptors does not influence MDPV effects on memory generalization. Together these findings indicate that the generalization effect induced by amphetamine is strongly regulated by the dopaminergic system, whereas the MDPV effects on memory generalization seem to be due to a selective activation of the noradrenergic system. Although these results require further investigation, it can be hypothesized that there is a differential recruitment induced by amphetamine and MDPV on the monoamine systems in different brain areas.

Brain regions with high density of DAT and dopaminergic receptors, such as the striatum and nucleus accumbens (Efimova et al., 2016) may be responsible for regulating amphetamine effects on memory generalization. Conversely, it is possible that the effects of MDPV on memory generalization are linked to brain areas with high levels of NET and β-adrenoceptors such as the dentate gyrus of the hippocampus and the perirhinal cortex, which are known to play a critical role in the regulation of memory discrimination (Miranda et al., 2017; van Dijk and Fenton, 2018). In agreement with these results, it could be hypothesized that the generalization induced

by MDPV is mediated by β-adrenoceptors in such brain areas. Thus, our findings demonstrate that both amphetamine and MDPV induce generalization of fear memory via a different involvement of NE and DA neurotransmission. These results pave the way for future studies aimed at investigating the role of specific brain areas in mediating the differential effects of both psychostimulant drugs on strength and quality of memory, thus ultimately leading to reveal the neurobiological underpinnings of memory alterations induced by drugs of abuse.

References

Asok, A., Kandel, E. R., and Rayman, J. B. (2018). The neurobiology of fear generalization. Front. Behav. Neurosci. 12:329. doi: 10.3389/fnbeh.2018.00329

Atehortua-Martinez, L. A., Masniere, C., Campolongo, P., Chasseigneaux, S., Callebert, J., Zwergel, C., et al. (2019). Acute and chronic neurobehavioral effects of the designer drug and bath salt constituent 3,4-methylenedioxypyrovalerone in the rat. J. Psychopharmacol. 33, 392–405. doi: 10.1177/0269881118822151

Atucha, E., and Roozendaal, B. (2015). The inhibitory avoidance discrimination task to investigate accuracy of memory. Front. Behav. Neurosci. 9:60. doi: 10.3389/fnbeh.2015.00060

Atucha, E., Vukojevic, V., Fornari, R. V., Ronzoni, G., Demougin, P., Peter, F., et al. (2017). Noradrenergic activation of the basolateral amygdala maintains hippocampus-dependent accuracy of remote memory. Proc. Natl. Acad. Sci. U S A 114, 9176–9181. doi: 10.1073/pnas.1710819114

Ballard, M. E., Gallo, D. A., and de Wit, H. (2012). Psychoactive drugs and false memory: comparison of dextroamphetamine and delta-9-tetrahydrocannabinol on false recognition. Psychopharmacology 219, 15–24. doi: 10.1007/s00213-011-2374-5

Baumann, M. H., Bukhari, M. O., Lehner, K. R., Anizan, S., Rice, K. C., Concheiro, M., et al. (2017). Neuropharmacology of 3,4-Methylenedioxypyrovalerone (MDPV), its metabolites and related analogs. Curr. Top. Behav. Neurosci. 32, 93–117. doi: 10.1007/7854_2016_53

Baumann, M. H., Partilla, J. S., Lehner, K. R., Thorndike, E. B., Hoffman, A. F., Holy, M., et al. (2013). Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive ‘‘bath salts’’ products. Neuropsychopharmacology 38, 552–562. doi: 10.1038/npp.2012.204

Bøhling, F. (2017). Psychedelic pleasures: an affective understanding of the joys of tripping. Int. J. Drug Policy 49, 133–143. doi: 10.1016/j.drugpo.2017.07.017

Carter, L. P., Kleykamp, B. A., Griffiths, R. R., and Mintzer, M. Z. (2013). Cognitive effects of intramuscular ketamine and oral triazolam in healthy volunteers. Psychopharmacology 226, 53–63. doi: 10.1007/s00213-012-2883-x

Colon-Perez, L. M., Tran, K., Thompson, K., Pace, M. C., Blum, K., Goldberger, B. A., et al. (2016). The psychoactive designer drug and bath salt constituent MDPV causes widespread disruption of brain functional connectivity. Neuropsychopharmacology 41, 2352–2365. doi: 10.1038/npp. 2016.40

Easton, C. J., and Bauer, L. O. (1997). Beneficial effects of thiamine on recognition memory and P300 in abstinent cocaine-dependent patients. Psychiatry Res. 70, 165–174. doi: 10.1016/s0165-1781(97)00024-3

Efimova, E. V., Gainetdinov, R. R., Budygin, E. A., and Sotnikova, T. D. (2016). Dopamine transporter mutant animals: a translational perspective. J. Neurogenet. 30, 5–15. doi: 10.3109/01677063.2016.1144751

Eiden, L. E., and Weihe, E. (2011). VMAT2: a dynamic regulator of brain monoaminergic neuronal function interacting with drugs of abuse. Ann. N. Y. Acad. Sci. 1216, 86–98. doi: 10.1111/j.1749-6632.2010.05906.x

Ferry, B., Parrot, S., Marien, M., Lazarus, C., Cassel, J. C., and McGaugh, J. L. (2015). Noradrenergic influences in the basolateral amygdala on inhibitory avoidance memory are mediated by an action on alpha2-adrenoceptors. Psychoneuroendocrinology 51, 68–79. doi: 10.1016/j.psyneuen.2014.09.010

Fleckenstein, A. E., Volz, T. J., Riddle, E. L., Gibb, J. W., and Hanson, G. R. (2007). New insights into the mechanism of action of amphetamines. Annu. Rev. Pharmacol. Toxicol. 47, 681–698. doi: 10.1146/annurev.pharmtox.47.120505. 105140

Gallagher, M., Kapp, B. S., Musty, R. E., and Driscoll, P. A. (1977). Memory formation: evidence for a specific neurochemical system in the amygdala. Science 198, 423–425. doi: 10.1126/science.20664

Gold, P. E., Edwards, R. M., and McGaugh, J. L. (1975). Amnesia produced by unilateral, subseizure, electrical stimulation of the amygdala in rats. Behav. Biol. 15, 95–105. doi: 10.1016/s0091-6773(75)92131-8

Gold, P. E., and van Buskirk, R. (1978). Posttraining brain norepinephrine concentrations: correlation with retention performance of avoidance training and with peripheral epinephrine modulation of memory processing. Behav. Biol. 23, 509–520. doi: 10.1016/s0091-6773(78)91614-0

Goodman, J., and Packard, M. G. (2016). Memory systems and the addicted brain. Front. Psychiatry 7:24. doi: 10.3389/fpsyt.2016.00024

Horry, R., Colton, L. M., and Williamson, P. (2014). Confidence-accuracy resolution in the misinformation paradigm is influenced by the availability of source cues. Acta Psychol. 151,

164–173. doi: 10.1016/j.actpsy.2014. 06.006

Hoscheidt, S. M., LaBar, K. S., Ryan, L., Jacobs, W. J., and Nadel, L. (2014). Encoding negative events under stress: high subjective arousal is related to accurate emotional memory despite misinformation exposure. Neurobiol. Learn. Mem. 112, 237–247. doi: 10.1016/j.nlm.2013.09.008

Karila, L., Megarbane, B., Cottencin, O., and Lejoyeux, M. (2015). Synthetic cathinones: a new public health problem. Curr. Neuropharmacol. 13, 12–20. doi: 10.2174/1570159x13666141210224137

Kjellgren, A., and Soussan, C. (2011). Heaven and hell--a phenomenological study of recreational use of 4-HO-MET in Sweden. J. Psychoactive Drugs 43, 211–219. doi: 10.1080/02791072.2011.605699

Koob, G. F. (2017). Antireward, compulsivity and addiction: seminal contributions of Dr. Athina Markou to motivational dysregulation in addiction. Psychopharmacology 234, 1315–1332. doi: 10.1007/s00213-016-4484-6

Koriat, A., Goldsmith, M., and Pansky, A. (2000). Toward a psychology of memory accuracy. Annu. Rev. Psychol. 51, 481–537. doi: 10.1146/annurev.psych. 51.1.481

Kutlu, M. G., and Gould, T. J. (2016). Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: contributions to development and maintenance of addiction. Learn. Mem. 23, 515–533. doi: 10.1101/lm.042192.116

LaLumiere, R. T., McGaugh, J. L., and McIntyre, C. K. (2017). Emotional modulation of learning and memory: pharmacological implications. Pharmacol. Rev. 69, 236–255. doi: 10.1124/pr.116.013474

LaLumiere, R. T., Nawar, E. M., and McGaugh, J. L. (2005). Modulation of memory consolidation by the basolateral amygdala or nucleus accumbens shell requires concurrent dopamine receptor activation in both brain regions. Learn. Mem. 12, 296–301. doi: 10.1101/lm.93205

Liang, K. C., Juler, R. G., and McGaugh, J. L. (1986). Modulating effects of posttraining epinephrine on memory: involvement of the amygdala noradrenergic system. Brain Res. 368, 125–133. doi: 10.1016/0006- 8993(86)91049-8

Liu, C. H., Ren, J., and Liu, P. K. (2016). Amphetamine manipulates monoamine oxidase-A level and behavior using theranostic aptamers of transcription factors AP-1/NF-kB. J. Biomed. Sci. 23:21. doi: 10.1186/s12929-016-0239-2

Loftus, E. F. (2005). Planting misinformation in the human mind: a 30-year investigation of the malleability of memory. Learn. Mem. 12, 361–366. doi: 10.1101/lm.94705

Martinez, J. L.Jr., Ishikawa, K., Liang, K. C., Jensen, R. A., Bennett, C., Sternberg, D. B., et al. (1983). 4-OH amphetamine enhances retention of an active avoidance response in rats and

decreases regional brain concentrations of norepinephrine and dopamine. Behav. Neurosci. 97, 962–969. doi: 10.1037//0735-7044.97.6.962

Martinez, J. L.Jr., Jensen, R. A., Messing, R. B., Vasquez, B. J., Soumireu- Mourat, B., Geddes, D., et al. (1980a). Central and peripheral actions of amphetamine on memory storage. Brain Res. 182, 157–166. doi: 10.1016/0006-8993(80)90838-0

Martinez, J. L.Jr., Vasquez, B. J., Rigter, H., Messing, R. B., Jensen, R. A., Liang, K. C., et al. (1980b). Attenuation of amphetamine-induced enhancement of learning by adrenal demedullation. Brain Res. 195, 433–443. doi: 10.1016/0006-8993(80)90077-3

Marusich, J. A., Antonazzo, K. R., Wiley, J. L., Blough, B. E., Partilla, J. S., and Baumann, M. H. (2014). Pharmacology of novel synthetic stimulants structurally related to the ‘‘bath salts’’ constituent 3,4-methylenedioxypyrovalerone (MDPV). Neuropharmacology 87, 206–213. doi: 10.1016/j.neuropharm.2014.02.016

McGaugh, J. L. (1973). Drug facilitation of learning and memory. Annu. Rev. Pharmacol. 13, 229–241. doi: 10.1146/annurev.pa.13.040173.001305

McGaugh, J. L., and Roozendaal, B. (2009). Drug enhancement of memory consolidation: historical perspective and neurobiological implications. Psychopharmacology 202, 3–14. doi: 10.1007/s00213-008-1285-6

McHugh, R. K., and Kneeland, E. T. (2019). Affective vulnerability in substance use disorders. Curr. Opin. Psychol. 30, 54–58. doi: 10.1016/j.copsyc.2019. 01.011

McIntyre, C. K., Power, A. E., Roozendaal, B., and McGaugh, J. L. (2003). Role of the basolateral amygdala in memory consolidation. Ann. N. Y. Acad. Sci. 985, 273–293. doi: 10.1111/j.1749-6632.2003.tb07088.x

Miller, H. H., Shore, P. A., and Clarke, D. E. (1980). in vivo monoamine oxidase inhibition by d-amphetamine. Biochem. Pharmacol. 29, 1347–1354. doi: 10.1016/0006-2952(80)904293

Miranda, M., Kent, B. A., Morici, J. F., Gallo, F., Weisstaub, N. V., Saksida, L. M., et al. (2017). Molecular mechanisms in perirhinal cortex selectively necessary for discrimination of overlapping memories, but independent of memory persistence. eNeuro 4:ENEURO.0293-17.2017. doi: 10.1523/eneuro.0293-17.2017

Móró, L., Simon, K., Bárd, I., and Rácz, J. (2011). Voice of the psychonauts: coping, life purpose and spirituality in psychedelic drug users. J. Psychoactive Drugs 43, 188–198. doi: 10.1080/02791072.2011.605661

Oeberst, A., and Blank, H. (2012). Undoing suggestive influence on memory: the reversibility of the eyewitness misinformation effect. Cognition 125, 141–159. doi: 10.1016/j.cognition.2012.07.009

Prosser, J. M., and Nelson, L. S. (2012). The toxicology of bath salts: a review of synthetic cathinones. J. Med. Toxicol. 8, 33–42. doi: 10.1007/s13181-011-0193-z

Quaedflieg, C. W. E. M., and Schwabe, L. (2018). Memory dynamics under stress. Memory 26, 364–376. doi: 10.1080/09658211.2017.1338299

Robertson, S. D., Matthies, H. J., and Galli, A. (2009). A closer look at amphetamine-induced reverse transport and trafficking of the dopamine and norepinephrine transporters. Mol. Neurobiol. 39, 73–80. doi: 10.1007/s12035-009-8053-4

Rogan, M. T., Stäubli, U. V., and LeDoux, J. E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. Nature 390, 604–607. doi: 10.1038/37601

Rohrbaugh, M., and Riccio, D. C. (1968). Stimulus generalization of learned fear in infant and adult rats. J. Comp. Physiol. Psychol. 66, 530–533. doi: 10.1037/h0026366

Roozendaal, B., Carmi, O., and McGaugh, J. L. (1996). Adrenocortical suppression blocks the memory-enhancing effects of amphetamine and epinephrine. Proc. Natl. Acad. Sci. U S A 93, 1429–1433. doi: 10.1073/pnas.93.4.1429

Roozendaal, B., Castello, N. A., Vedana, G., Barsegyan, A., and McGaugh, J. L. (2008). Noradrenergic activation of the basolateral amygdala modulates consolidation of object recognition memory. Neurobiol. Learn. Mem. 90, 576–579. doi: 10.1016/j.nlm.2008.06.010

Roozendaal, B., de Quervain, D. J., Schelling, G., and McGaugh, J. L. (2004). A systemically administered beta-adrenoceptor antagonist blocks corticosteroneinduced impairment of contextual memory retrieval in rats. Neurobiol. Learn. Mem. 81, 150–154. doi: 10.1016/j.nlm.2003.10.001

Ruediger, S., Vittori, C., Bednarek, E., Genoud, C., Strata, P., Sacchetti, B., et al. (2011). Learning-related feedforward inhibitory connectivity growth required for memory precision. Nature 473, 514–518. doi: 10.1038/nature09946

Schwabe, L. (2017). Memory under stress: from single systems to network changes. Eur. J. Neurosci. 45, 478–489. doi: 10.1111/ejn.13478

Simmler, L. D., Buser, T. A., Donzelli, M., Schramm, Y., Dieu, L. H., Huwyler, J., et al. (2013). Pharmacological characterization of designer cathinones in vitro. Br. J. Pharmacol. 168, 458–470. doi: 10.1111/j.1476-5381.2012.02145.x

Teng, L., Crooks, P. A., and Dwoskin, L. P. (1998). Lobeline displaces [3H]dihydrotetrabenazine binding and releases [3H]dopamine from rat striatal synaptic vesicles: comparison with d-amphetamine. J. Neurochem. 71, 258–265. doi: 10.1046/j.1471-4159.1998.71010258.x

Trost, A., and Hauber, W. (2014). Dopamine D1/D2 receptors do not mediate the expression of conditioned place preference induced by the aftereffect of wheelm running. BMC Neurosci. 15:124. doi: 10.1186/s12868-014-0124-4

Tyng, C. M., Amin, H. U., Saad, M. N. M., and Malik, A. S. (2017). The influences of emotion on learning and memory. Front. Psychol. 8:1454. doi: 10.3389/fpsyg. 2017.01454

van Dijk, M. T., and Fenton, A. A. (2018). On how the dentate gyrus contributes to memory discrimination. Neuron 98, 832.e835–845.e835. doi: 10.1016/j.neuron. 2018.04.018

Wideman, C. E., Jardine, K. H., and Winters, B. D. (2018). Involvement of classical neurotransmitter systems in memory reconsolidation: focus on destabilization. Neurobiol. Learn. Mem. 156, 68–79. doi: 10.1016/j.nlm.2018.11.001

Xu, W., and Sudhof, T. C. (2013). A neural circuit for memory specificity and generalization. Science 339, 1290–1295. doi: 10.1126/science.1229534

CHAPTER 2

KETAMINE ANESTHESIA ENHANCES FEAR MEMORY CONSOLIDATION VIA NORADRENERGIC ACTIVATION IN THE BASOLATERAL AMYGDALA

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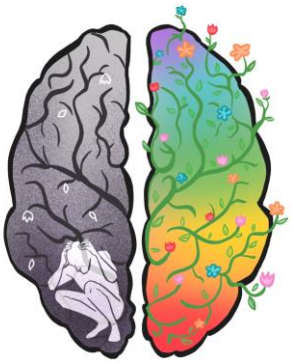

Abstract

Trauma patients treated with ketamine during emergency care present aggravated early post- traumatic stress reaction which is highly predictive of post-traumatic stress disorder (PTSD) development and severity. The use of ketamine in the acute trauma phase may directly or indirectly interfere with neural processes of memory consolidation of the traumatic event, thus leading to the formation of maladaptive memories, a hallmark symptom of PTSD. We have recently shown that ketamine anesthesia, immediately after a traumatic event, enhances memory consolidation and leads to long-lasting alterations of social behavior in rats. Based on the evidence that ketamine induces a robust central and peripheral adrenergic/noradrenergic potentiation and that activation of this system is essential for the formation of memory for stressful events, we explored the possibility that the strong sympathomimetic action of ketamine might underlie its memory enhancing effects. We found that rats given immediate, but not delayed, post-training ketamine anesthesia (125 mg/kg) presented enhanced 48-h memory retention in an inhibitory avoidance task and that these effects were blocked by adrenal medullectomy, lesions of the locus coeruleus, systemic or intra-basolateral amygdala ß-adrenergic receptor antagonism. Thus, the memory enhancing effects of ketamine anesthesia are time-dependent and mediated by a combined peripheral-central sympathomimetic action. We elucidated a mechanism by which ketamine exacerbates acute post-traumatic reaction, possibly leading to development of PTSD symptomatology later in life. These findings will help guide for a better management of sedation/anesthesia in emergency care to promote the prophylaxis and reduce the risk of developing trauma-related disorders in trauma victims.

Introduction

Trauma victims present increased risk of developing post-traumatic stress disorder (PTSD), characterized, among other symptoms, by altered memory processing of the traumatic experience (American Psychiatric Association, 2013). Drugs used in emergency care can interfere with memory formation for the traumatic event, and thus influence the risk to develop PTSD later in life. Ketamine is an intravenous anesthetic commonly used in clinics, particularly in emergency care, for premedication, sedation, induction and maintenance of general anesthesia, in children and adults (Green et al., 1998; Weant, Davis, Davis, & Hooper, 2019). Ketamine is primarily known as a NMDA glutamate receptor antagonist (Harrison & Simmonds, 1985). However, it has several off-site targets (Sleigh, Harvey, Voss, & Denny, 2014; Zanos et al., 2016) mediating different effects. Ketamine's ability to increase heart rate and blood pressure and alter attentional processes has been attributed to its sympathomimetic action (Baraka, Harrison, & Kachachi, 1973), achieved through blockade of both peripheral and central adrenaline/noradrenaline re-uptake (Hara et al., 1998; White, Way, & Trevor, 1982; Zhao & Sun, 2008). Furthermore, ketamine increases the firing rate of noradrenergic neurons in the locus coeruleus (LC), the primary source of noradrenaline in the central nervous system (CNS), through activation of AMPA receptors (El Iskandrani, Oosterhof, El Mansari, & Blier, 2015). It is well established that during stressful events adrenaline is released from the adrenal medulla and, through the vagus nerve, activates the central noradrenergic system in the nucleus of the solitary tract and LC, which in turn triggers noradrenergic mechanisms in the basolateral amygdala (BLA) resulting in enhanced memory for these events (Atsak et al., 2015; McGaugh, 2000; McIntyre, Hatfield, & McGaugh, 2002; Quirarte, Roozendaal, & Mcgaugh, 1997; Roozendaal et al., 2006). As trauma victims and patients in emergency care often experience stressful events shortly before ketamine administration, it is of crucial importance to investigate its effects, since the immediate post-trauma phase is characterized by a rapid surge of peripheral and central

adrenaline/noradrenaline levels and the traumatic memory is consolidated into a stable long-term memory trace. Excessive consolidation of such events might lead to PTSD development (Pitman et al., 2012). Evidence exists that ketamine modulates or aggravates early post-traumatic stress reactions when given in the acute trauma phase, and it correlates with onset of sustained PTSD symptomatology (M. Schönenberg, Reichwald, Domes, Badke, & Hautzinger, 2008; Michael Schönenberg, Reichwald, Domes, Badke, & Hautzinger, 2005; Winter & Irle, 2004). However, recent findings indicate that ketamine, at sub-anesthetic doses, improves psychiatric symptoms in individuals already suffering from PTSD and comorbid depression (Albott et al., 2018; Hartberg, Garrett-Walcott, & De Gioannis, 2018). This suggests that ketamine might increase the risk of PTSD development when given in close proximity of the trauma, plausibly by potentiating the noradrenergic-mediated enhancement of memory consolidation. Interestingly, treatment with the β-adrenoceptor antagonist propranolol, although not always effective, reduces fear and PTSD development when paired with behavioral therapy soon after trauma and ameliorates the symptomatology when PTSD is already developed (Giustino, Fitzgerald, & Maren, 2016). Furthermore, reduction of noradrenergic release with dexmedetomidine, an α2-adrenoceptor agonist, decreases the duration of delirium in intensive or post-anesthesia care unit, or after cardiac surgery (Pandharipande et al., 2007; Read, Maani, & Blackwell, 2017; Shehabi et al., 2009). Accordingly, in rats, we have shown that dexmedetomidine reduces, while ketamine anesthesia enhances memory retention and exacerbates PTSD symptomatology, when injected soon after a traumatic event (Morena et al., 2017). Here, we examined whether ketamine memory enhancing effects were specific for the early phases of consolidation by testing the effects of post-training immediate, or delayed, ketamine anesthesia on inhibitory avoidance memory retention in rats. Then, we explored the contribution of the adrenergic system in mediating such effects by injecting ketamine in adrenal medullectomized, or LC lesioned rats, or in the presence of a systemic, or intra-BLA, injection of propranolol.

Materials and methods

Animals

A total of two hundred and six male naïve Sprague-Dawley rats (350–450 g at the time of behavioral experiments; Charles River Laboratories, Calco, Italy) were included in the present investigation. Animals were kept in an air-conditioned controlled room (temperature: 21°±1°C; lights on 7:00 A.M.–7:00 P.M.) with food and water available ad libitum. Training and testing were performed during the light phase of the cycle between 10:00 A.M. and 3:00 P.M. All procedures were in compliance with the Guide for the Care and Use of Laboratory Animals, the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, and the D. L. 26/2014 of Italian Ministry of Health.

Drug treatment

Propranolol hydrochloride (Tocris, Milan, Italy), when injected systemically, and ketamine (Ratiopharm, Ulm, Germany) were dissolved, or diluted, respectively, in saline solution (0.9%). Lidocaine (Tocris, Milan, Italy) and propranolol hydrochloride (Tocris, Milan, Italy), when injected centrally, were dissolved in phosphate buffered saline 1X (PBS, pH = 7.4). Drug solutions were freshly prepared the day of the experiment. Ketamine (125 mg/kg) was administered by intraperitoneal injection (i.p.) immediately or 3h after the inhibitory avoidance training trial. Propranolol (2 mg/kg) was given i.p. 30 min before training. In a separate experiment, propranolol (0.5 μg/0.2 μl/side) was administered bilaterally into the BLA immediately after the inhibitory avoidance training. Lidocaine (4% wt/vol; 4 g/100 ml of PBS) was administered bilaterally into the LC immediately after the inhibitory avoidance training. Drug doses were chosen on the basis of previous studies performed in our laboratory and by others (Campolongo, Roozendaal, Trezza, Cuomo, et al., 2009; Lashgari, Khakpour-Taleghani, Motamedi, & Shahidi, 2008; Morena et al., 2017; Quirarte et al., 1997; Roozendaal, McReynolds, & McGaugh, 2004; Song, Ansah, Meyerson, Pertovaara,

& Linderoth, 2013). Post-training central infusions of drugs or their respective vehicles were made by using a 30-gauge injection needle connected by polyethylene tubing (PE-20) to a 10 μL Hamilton microsyringe driven by a minipump (KD Instruments, Canning Vale, Australia). The injection needles protruded 2.0 mm beyond the tip of the cannula, and a 0.2 μL, or 0.5 μl, injection volume per hemisphere was infused over a period of 25–85 s in the BLA, or LC, respectively. The injection needles were retained within the cannulae for an additional 20 s after infusion to maximize diffusion and to prevent backflow of drug into the cannulae. The infusion volumes were chosen on the basis of previous experiments (Guo, Jiang, Buttermann, & Maze, 1996; Morena et al., 2019).

Cannulation

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), given atropine sulfate (0.4 mg/kg, i.p.) to maintain respiration, and subsequently injected with 3 mL of saline subcutaneously to facilitate clearance of drugs and prevent dehydration. The rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), and two stainless-steel guide cannulae (23-gauge) were implanted bilaterally, with the cannula tips 2 mm above the BLA [15 mm-long; coordinates: anteroposterior (AP), −2.8 mm from Bregma; mediolateral (ML), ±5.0 mm from midline; dorsoventral (DV), −6.5 mm from skull surface] or 2 mm above the LC [13 mm-long; coordinates: AP, −9.8 mm; ML, ±1.2 mm; DV, −5.1 mm](Guo et al., 1996; Morena et al., 2016). Stylets (15 mm- or 13 mm-long 00 insect dissection pins) were inserted into each cannula to maintain patency. Rats were allowed to recover from surgery for at least 1 week before training and handled three times for 1 min before training.

Adrenal medullectomy

Adrenal medullectomy (ADMX) was performed as previously described (Ferrari et al.,

2010; Khasar et al., 1998; Wilkinson et al., 1981). Briefly, rats were anesthetized with isoflurane (2.5% isoflurane in O_2). Each animal was placed on a flat surface with their limbs in the extended position and their dorsal area was trichotomized. Incisions of 2 cm were made on the right and left dorsal lateral surface of the animals just over each kidney. Small incisions were made on the adrenal capsule and the medulla were removed. Sham ADMX rats only received small skin incisions. Wounds were closed with suture clips. Rats were allowed to recover from surgery for 5 weeks before starting behavioral experiments. ADMX rats were given 0.50% saline to drink in place of water for 1 week after surgery to compensate for salt and water losses during the period of corticosteroid deficiency (Khasar et al., 1998; Wilkinson et al., 1981).

Inhibitory avoidance test

Rats were trained and tested in an inhibitory avoidance apparatus as previously described (Morena et al., 2017; Ratano et al., 2018). Briefly, the apparatus consisted of a trough-shaped alley divided into two compartments, separated by a sliding door. The starting compartment was made of opaque white plastic and illuminated; the shock compartment was made of two dark, electrifiable metal plates and was not illuminated. Animals were handled 1 min each for 3 days before training. For training, the rats were placed into the starting compartment of the apparatus, after rats stepped completely into the dark compartment, the sliding door was closed and a single, inescapable footshock (0.35 mA) was delivered for 1 s. Cannulated rats received higher footshock intensity (0.60 mA for LC cannulation and 0.65 mA for BLA cannulation) to ensure a good memory performance in all experimental groups (Campolongo et al., 2009; Morena et al., 2014; Wotjak, 2019). The animals were removed from the shock compartment 15 s after the footshock termination. Retention was tested 48-h later. On the retention test, rats were placed into the starting compartment, and the latency to reenter the shock compartment with all four paws (retention latency; 600 s cut-off) was recorded. Longer latencies were interpreted as indicating better memory retention (McGaugh and Dawson, 1971). After each session, the apparatus was cleaned with a 70% ethanol

solution.

Sleep parameters

Sleep onset time and duration for rats given ketamine were measured as previously described (Hauer et al., 2011; Morena et al., 2017). Immediately after the anesthetic administration each rat was placed on its back once every 30 s until it was unable to right itself within 30 s. Sleep onset time was defined as the interval between anesthetic injection and the time the rat was unable to turn itself upright. The time between loss and recovery of righting reflex for each rat was defined as sleep duration (180 min cut-off). The loss of righting reflex was defined when the rat, after ketamine injection, was unable to turn itself upright at least twice within 1 min. Then, each rat was left undisturbed on its back until it spontaneously regained its righting reflexes, defined as having at least three paws under its body. Complete recovery of the righting reflex was defined as the rat being able to turn itself upright.

Histology

To check for cannula placement, cannulated rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 0.9% saline. The brains were then removed and immersed in a 4% (wt/vol) formaldehyde solution. At least 48 h before sectioning, the brains were transferred to a 20% (wt/vol) sucrose solution in saline for cryoprotection. Coronal sections of 35 μm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. The sections were examined under a light microscope (Nikon 801 microscope, Nikon instruments SPA, Milan, Italy) and the location of infusion needle tips in the BLA or LC were made by an observer blind to treatment conditions.

Statistical analysis

The person who performed and analyzed the test was blinded to the condition of the animals. Statistical analysis was performed using GraphPad Prism Software (La Jolla,

CA, USA). To determine whether learning had occurred in the inhibitory avoidance training, paired t-tests were used to compare the training and retention latencies of vehicle control groups. Sleep duration was analyzed with unpaired t-tests and correlation analyses were performed with the Pearson correlation test. For the other behavioral measures, unpaired t tests or two-way ANOVAs with the treatments as between-subjects factors, followed by Tukey-Kramer post-hoc tests, were used when appropriate. Data are expressed as mean ± standard error of the mean (SEM). P values of < 0.05 were considered statistically significant. The number of rats per group is indicated in the figure legends. A total of 6 rats were excluded from statistical analyses for cannula misplacement.

Results

Immediate post-training, but not delayed, ketamine injection enhances aversive memory retention

We have previously shown that an anesthetic dose of ketamine (125 mg/kg, i.p.) given immediately after an inhibitory avoidance training enhances 48-h memory retention in rats (Morena et al., 2017). To examine whether ketamine specifically enhances the early consolidation phase of aversive memory processing, rats were treated with ketamine, or its vehicle, immediately, or 3h after the inhibitory avoidance training (experiment 1; Fig. 1A). By replicating our previous results (Morena et al., 2017), we found that immediate post-training ketamine injection enhanced 48-h memory retention (Fig. 1B). Average step-through latencies for all groups during training, before footshock and immediate post-training drug treatment, were 10.11 ± 1.36 s (mean ± SEM). Unpaired t test for training latencies did not reveal any significant difference between vehicle and ketamine groups injected immediately post-training ($t_{14} = 0.52$, $P = 0.61$), indicating that there were not pre-existing behavioral differences between the two experimental groups. Retention latencies of vehicle-treated rats were significantly longer than their training latencies ($t_7 = 2.68$, $P = 0.03$), indicating that rats correctly retained memory of the shock experience. As, shown in Figure 1B, unpaired t test revealed that rats given

immediate post-training ketamine injection presented significantly higher retention latencies than controls (t_{14} = 2.51, P = 0.03). Average step-through latencies during training, before footshock, for vehicle and ketamine groups injected 3-h post-training, were 9.54 ± 1.12 s (mean ± SEM). Unpaired t test for training latencies did not reveal any significant difference between vehicle and ketamine groups injected 3-h post-training (t_{14} = 1.97, P = 0.07), showing no pre-existing differences between groups before drug treatment. Retention latencies of vehicle-treated rats were significantly longer than their training latencies (t_7 = 3.16, P = 0.02), indicating that rats correctly retained memory of the shock experience. Unpaired t test for retention latencies did not reveal any significant difference between vehicle- and ketamine-treated rats injected 3-h after training (t_{14} = 1.47, P = 0.16; Fig. 1C). These results show that ketamine anesthesia enhances 48-h retention latencies when given immediately, but not 3-h after training.

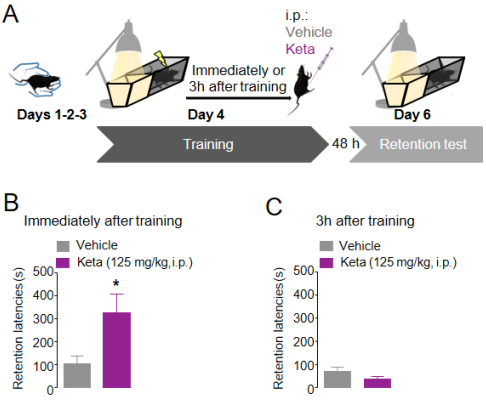

Figure 1: Immediate, but not delayed, post-training ketamine anesthesia enhances 48-h inhibitory avoidance memory retention. Schematic representation of experiment 1 (A). Ketamine injection (Keta; 125 mg/kg, i.p.) enhanced 48-h memory retention when injected immediately (B), but not 3-h after training (C). *, P < 0.05 vs vehicle group. Results represent mean ± SEM (n = 8 per group).

Memory enhancing effects of immediate post-training ketamine injection are dependent on adrenal catecholamines

To examine whether the memory enhancing effects of immediate post-training ketamine injection were dependent on adrenal catecholamines, the anesthetic agent, or its vehicle, was given to sham ADMX or ADMX rats immediately after the inhibitory avoidance training (experiment 2; Fig. 2A). Average step-through latencies for all groups during training, before footshock and drug treatment, were 13.34 ± 1.78 s (mean ± SEM). Two-way ANOVA for training latencies did not reveal any significant ketamine treatment ($F_{1,35} = 0.06$, $P = 0.82$), ADMX ($F_{1,35} = 2.38$, $P = 0.13$), or ketamine x ADMX interaction ($F_{1,35} = 0.01$, $P = 0.92$) effects, indicating that there were not pre-existing behavioral differences among all experimental groups. Retention latencies of sham ADMX rats treated with vehicle were significantly longer than their training latencies ($t_{12} = 3.09$, $P = 0.009$), indicating that rats correctly retained memory of the shock experience. As, shown in Figure 2C, two-way ANOVA for retention latencies revealed significant ketamine ($F_{1,35} = 9.54$, $P = 0.004$) and ADMX ($F_{1,35} = 12.86$, $P = 0.001$) effects and a significant interaction between both factors ($F_{1,35} = 7.49$, $P = 0.01$). Post-hoc tests indicated that retention latencies of sham ADMX rats treated with ketamine (125 mg/kg) were significantly longer than those of both sham ADMX rats given vehicle and ADMX rats treated with ketamine ($P < 0.001$ for both comparisons; Fig. 2C). There were no significant differences between sham ADMX and ADMX rats treated with vehicle. These results indicate that a functional adrenal medulla capable to release adrenaline in the blood stream after a fearful experience is required for enabling the enhancing effects of ketamine on fear memory consolidation.

Memory enhancing effects of immediate post-training ketamine injection are dependent on β-adrenoceptor activation

This experiment examined whether memory enhancing effects of immediate post-

training ketamine anesthesia were dependent on β-adrenoceptor activation (experiment 3; Fig. 2B). Immediate post-training injections of ketamine (125 mg/kg, i.p.), or its vehicle, were given to rats pre-treated with systemic injections of the β-adrenoceptor antagonist propranolol (2 mg/kg, i.p.), or its vehicle, 30 min before the inhibitory avoidance training. Average step-through latencies for all groups during training, before footshock and drug treatment, were 11.78 ± 1.37 s (mean ± SEM). Two-way ANOVA for training latencies did not reveal any significant ketamine ($F_{1,42}$ = 0.97, P = 0.33) or propranolol ($F_{1,42}$ = 0.49, P = 0.49) treatment effects or an interaction between both factors ($F_{1,42}$ = 1.69, P = 0.20). Retention latencies of vehicle-treated rats were significantly longer than their training latencies (t_{11} = 2.45, P = 0.03), indicating that rats retained memory of the shock experience. As shown in Figure 2D, two-way ANOVA for retention latencies revealed significant propranolol ($F_{1,42}$ = 9.05, P = 0.004) and ketamine x propranolol interaction ($F_{1,42}$ = 11.42, P = 0.002) effects, but no significant effect of ketamine ($F_{1,42}$ = 2.87, P = 0.10). Post-hoc tests indicated that retention latencies of rats treated with ketamine alone were significantly longer than those of both vehicle-treated rats (P < 0.01; Fig. 2D) and rats given propranolol together with ketamine (P < 0.001; Fig. 2D). There were no significant differences between rats treated with vehicle alone and rats given propranolol alone. These results indicate that memory enhancing effects of ketamine require indirect β-adrenoceptor activation.

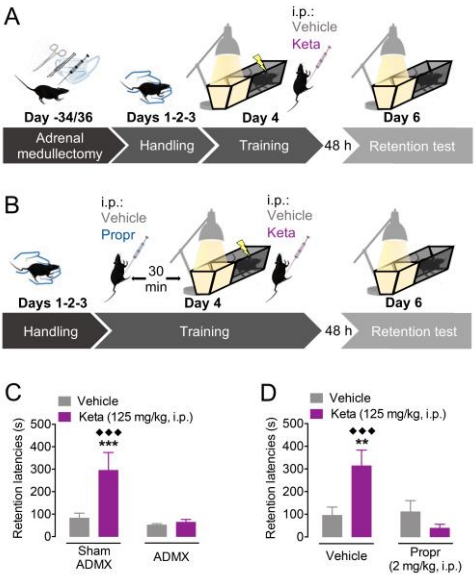

Figure 2: Systemic adrenergic blockade prevents ketamine enhancing effects on 48-h inhibitory avoidance retention. Schematic representations of experiment 2 (A) and experiment 3 (B). Immediate post-training administration of ketamine (Keta; 125 mg/kg, i.p.) enhanced memory retention and either adrenal medullectomy (ADMX) (C) or systemic □-adrenergic receptor antagonism with propranolol (Propr; 2 mg/kg, i.p.) (D) blocked the memory enhancing effects of post-training ketamine. **, $P < 0.01$; ***, $P < 0.001$ vs the corresponding vehicle group; •••, $P < 0.001$ vs the corresponding ADMX or propranolol group. Results represent mean ± SEM (n = 8 - 13 per group).

An intact locus coeruleus is required to enable memory enhancing effects of immediate post-training ketamine injection

To examine whether memory enhancing effects of immediate post-training ketamine anesthesia were dependent on central adrenergic activity, two separate groups of rats received either post-training bilateral sham LC lesions with vehicle or temporary LC lesions with lidocaine (4%, 0.5 µl/side) infusion. Immediately after LC infusions, each

group was also administered with either ketamine (125 mg/kg, i.p.), or its vehicle (experiment 4; Fig. 3A). Average step-through latencies for all groups during training, before footshock and drug treatment, were 7.56 ± 0.43 s (mean ± SEM). Two-way ANOVA for training latencies did not reveal any significant ketamine ($F_{1,43}$ = 2.60, P = 0.11), LC lesion ($F_{1,43}$ = 3.15, P = 0.08) or ketamine x LC lesion interaction ($F_{1,43}$ = 2.51, P = 0.12) effects. Retention latencies of sham lesioned vehicle-treated rats were significantly longer than their training latencies (t_{14} = 2.15, P = 0.04), indicating that rats retained memory of the shock experience. As shown in Figure 3C, two-way ANOVA for retention latencies revealed significant ketamine ($F_{1,43}$ = 4.84, P = 0.03) and LC lesion ($F_{1,43}$ = 5.25, P = 0.03) effects, but no significant interaction between both factors ($F_{1,43}$ = 2.98, P = 0.09). Post-hoc analyses indicated that retention latencies of sham lesioned rats treated with ketamine were significantly longer than those of both sham lesioned vehicle-treated rats and LC lesioned rats given ketamine (P < 0.05, for both comparisons; Fig. 3C). There were not significant differences between sham lesioned and LC lesioned rats treated with vehicle. These results indicate that memory enhancing effects of ketamine require central noradrenaline release. Diagrams in Figure 3D show rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis.

Memory enhancing effects of immediate post-training ketamine injection are dependent on β-adrenoceptor activation in the BLA

This experiment examined whether memory enhancing effects of immediate post-training ketamine injection were dependent on β-adrenergic receptor activation in the BLA (experiment 5; Fig. 3B). Post-training injections of ketamine (125 mg/kg, i.p.), or its vehicle, were administered to rats given bilateral intra-BLA infusions of either propranolol (0.5 μg/0.2 μl/side), or its vehicle, immediately post-training and before ketamine injection. Average step-through latencies for all groups during training, before footshock and drug treatment, were 11.00 ± 1.12 s (mean ± SEM). Two-way ANOVA

for training latencies did not reveal any significant ketamine ($F_{1,38}$ = 1.14, P = 0.29) or propranolol ($F_{1,38}$ = 0.69, P = 0.41) treatment effects or an interaction between both factors ($F_{1,38}$ = 3.01, P = 0.09). Retention latencies of vehicle-treated rats were significantly longer than their training latencies (t_9 = 2.62, P = 0.03), indicating that rats retained memory of the shock experience. As shown in Figure 3E, two-way ANOVA for retention latencies revealed significant ketamine ($F_{1,38}$ = 6.11, P = 0.02), propranolol ($F_{1,38}$ = 7.63, P = 0.009) and ketamine x propranolol interaction ($F_{1,38}$ = 4.58, P = 0.04) effects. Post-hoc tests indicated that retention latencies of rats treated with ketamine alone were significantly longer than those of both vehicle alone-treated rats (P < 0.05; Fig. 3E) and rats given intra-BLA propranolol together with ketamine (P < 0.01; Fig. 3E). There were no significant differences between rats treated with vehicle alone and rats given propranolol alone. These results indicate that memory enhancing effects of ketamine require indirect β-adrenoceptor activation in the BLA. Diagrams in Figure 3F show rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis.

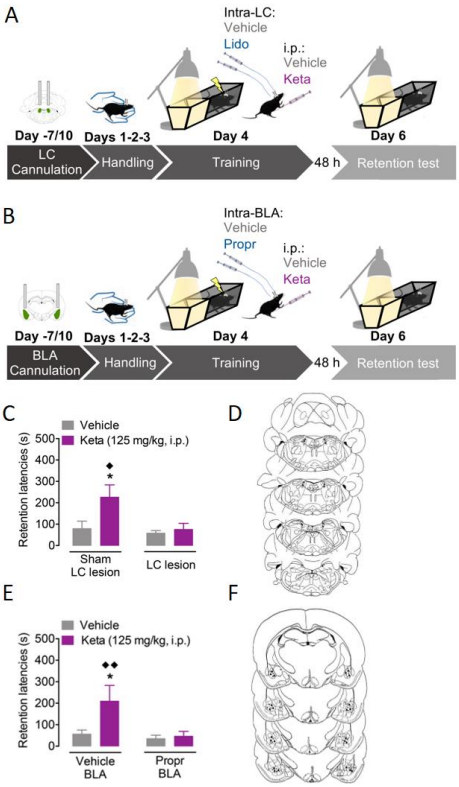

Figure 3: Central adrenergic blockade within the LC or BLA prevents ketamine enhancing effects on 48-h inhibitory avoidance retention. Schematic representations of experiment 4 (A) and experiment 5 (B). Immediate post-training LC temporary lesions (C) blocked ketamine (Keta; 125 mg/kg, i.p.) enhancing effects on memory retention. Diagrams on the right (D) show rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis (● Vehicle; □ LC lesion). Immediate post-training intra-BLA □-adrenergic receptor antagonism with propranolol (Propr; 0.5 µg/0.2 µl/side) (E) blocked ketamine (Keta; 125 mg/kg, i.p.) enhancing effects on memory retention. Diagrams on the right (F) show rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis (● Vehicle; ▲ Propr). *, $P < 0.05$ vs the corresponding vehicle group; •, $P < 0.05$; ••, $P < 0.01$ vs the corresponding LC lesioned group or intra-BLA propranolol group. Results represent mean ± SEM. Vehicle/Sham LC lesion, n = 15; Keta/Sham LC lesion, n = 11; Vehicle/LC lesion, n = 10;

Keta/LC lesion, n = 11; Vehicle/Vehicle BLA, n = 10; Keta/Vehicle BLA, n = 8; Vehicle/Propr BLA, n = 11; Keta/Propr BLA, n = 13.

Memory enhancing effects of post-training ketamine injection are not influenced by anesthesia-induced sleep parameters

Many studies have documented a close relationship between sleep and memory and have shown that sleep influences processes of memory consolidation (Rasch and Born, 2013). We examined whether the different experimental conditions used in our study altered the onset and/or the duration of ketamine-induced anesthesia and whether this influenced 48-h memory retention.

Table 1 shows sleep onset time and duration for rats given ketamine in all the experimental groups. Unpaired t-tests for sleep onset time of rats given ketamine (alone or in combination with other treatments) showed no significant differences between groups within each experiment ($t_{14} = 1.13$, $P = 0.28$; $t_{15} = 1.00$, $P = 0.33$; $t_{20} = 1.88$, $P = 0.08$; $t_{20} = 1.00$, $P = 0.33$; $t_{19} = 0.48$, $P = 0.64$; for experiment 1, 2, 3, 4 and 5, respectively; Table 1). Similarly, unpaired t-tests for sleep duration of rats given ketamine (alone or in combination with other treatments) showed no significant differences between groups within each experiment ($t_{14} = 1.32$, $P = 0.21$; $t_{15} = 0.12$, $P = 0.90$; $t_{20} = 1.69$, $P = 0.11$; $t_{20} = 0.45$, $P = 0.66$; $t_{19} = 0.89$, $P = 0.38$; for experiment 1, 2, 3, 4 and 5, respectively; Table 1), thus, showing that neither the different experimental conditions nor the treatments combined with ketamine altered sleep onset or duration induced by the anesthetic injection. Moreover, Pearson's analyses did not reveal any significant correlation between sleep onset time, or duration, with 48-h retention latencies shown by all rats treated with ketamine (Table 1), thus indicating that the memory enhancing effects of ketamine are not related to the anesthetic properties of the drug.

Table 1. Sleep parameters of ketamine-treated rats

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5	
Experimental group	Keta i.p. imm. post-training	Keta i.p. 3h post-training	Sham ADMX + Keta i.p.	ADMX + Keta i.p.	Veh i.p. + Keta i.p.	Propr i.p. + Keta i.p.	Sham LC lesion + Keta i.p.	LC lesion + Keta i.p.	Veh BLA + Keta i.p.	Propr BLA + Keta i.p.
Sleep onset time (min)	5.75 ± 1.28	3.88 ± 1.06	5.88 ± 1.30	10.00 ± 3.70	4.44 ± 0.65	6.23 ± 0.65	6.73 ± 1.53	5.09 ± 0.56	5.75 ± 1.00	6.54 ± 1.14
Correlation: Sleep onset time vs Retention latencies	r = -0.17, P = 0.69	r = -0.35, P = 0.40	r = 0.15, P = 0.73	r = -0.33, P = 0.38	r = -0.06, P = 0.88	r = 0.38, P = 0.21	r = 0.03, P = 0.92	r = 0.13, P = 0.70	r = 0.64, P = 0.09	r = -0.29, P = 0.34
Sleep duration (min)	42.25 ± 5.59	51.38 ± 4.08	39.13 ± 5.40	39.89 ± 3.29	45.11 ± 6.21	35.77 ± 1.77	48.64 ± 5.14	52.45 ± 6.75	40.38 ± 3.05	47.23 ± 5.69
Correlation: Sleep duration vs Retention latencies	r = -0.03, P = 0.95	r = 0.12, P = 0.77	r = -0.20, P = 0.63	r = 0.22, P = 0.56	r = 0.41, P = 0.27	r = 0.29, P = 0.33	r = -0.10, P = 0.76	r = -0.01, P = 0.98	r = -0.43, P = 0.29	r = -0.28, P = 0.36

Effects of intraperitoneal ketamine injections on rats' sleep onset time and duration tested under the different experimental conditions and Pearson's correlations analyses between sleep onset time, or duration, of ketamine-treated rats and their respective 48-h retention latencies. Experiment 1: Rats injected with ketamine, i.p., immediately after training (Keta i.p. imm. post-training) and rats injected with ketamine, i.p., 3h after training (Keta i.p. 3h post-training). Experiment 2: Sham adrenal medullectomized rats + ketamine i.p. (Sham ADMX + Keta i.p.) and adrenal medullectomized rats + ketamine i.p. (ADMX + Keta i.p.). Experiment 3: Rats injected with vehicle, i.p. 30 min before training and ketamine immediately after training (Veh i.p. + Keta i.p.) and rats injected with propranolol, i.p. 30 min before training and ketamine immediately after training (Propr i.p. + Keta i.p.). Experiment 4: Sham Locus Coeruleus lesioned rats + ketamine i.p. (Sham LC lesion + Keta i.p.) and Locus Coeruleus lesioned rats + ketamine i.p. (LC lesion + Keta i.p.). Experiment 5: Rats given intra-basolateral amygdala vehicle injection + ketamine i.p. (Veh BLA + Keta i.p.) and rats given intra-basolateral amygdala propranolol injection + ketamine i.p. (Propr BLA + Keta i.p.). Data are expressed as mean ± SEM (n =8 - 13 per group).

Discussion

The present findings indicate that ketamine anesthesia enhances memory consolidation of fearful experiences when administered shortly after an inhibitory avoidance training via a combined peripheral and central sympathomimetic action which likely terminates with the activation of the BLA noradrenergic system. We have shown that ketamine anesthesia strengthens the traumatic memory trace when given immediately after a stressful event and induces pronounced and persistent emotional dysfunction in rats, by reducing social interaction with a conspecific, sixteen days after trauma and ketamine injection (Morena et al., 2017). By adding to our previous findings, here we unveiled a possible mechanism underlying the traumatic memory enhancing properties of ketamine anesthesia by showing that these effects are time-dependent and arise from the strong sympathomimetic action of the anesthetic agent. Specifically, our results reveal that ketamine anesthesia modulates time-dependent processes of aversive memory consolidation together with a combined peripheral and central action of ketamine in potentiating adrenergic/noradrenergic signaling to promote aversive memory retention. Adrenal medullectomy or blockade of ß-adrenoceptors, with systemic injections of propranolol, although not altering 48-h memory retention *per se*, prevented the memory potentiating effects of immediate post-training ketamine injection. Furthermore, our findings show that temporary post-training lesions of the LC completely blocked the memory enhancing effects of ketamine. Our last experiment identified the BLA as a candidate brain area responsible for ketamine-mediated enhancement of traumatic memory retention. Selective blockade of ß-adrenoceptors within the BLA completely prevented the effects of post-training ketamine anesthesia. The sympathomimetic action of ketamine, via inhibition of the noradrenaline transporter, at both the peripheral and central levels, or by increasing LC noradrenergic neuronal firing, has been widely demonstrated (El Iskandrani et al., 2015; Hara et al., 1998; Nishimura et al., 1998; Zhao and Sun, 2008). The LC represents a main source of noradrenaline in the CNS (Mouton et al., 1994) and sends dense projections to nearly the whole brain, including the BLA (Asan, 1998), which is engaged when fear stimuli

elicit an arousal effect to modulate anxiety and processes of fear memory formation (Atsak et al., 2015; McGaugh, 2015; McIntyre et al., 2002; Morena et al., 2016). Increased noradrenergic release in the rat BLA has been observed during an inhibitory avoidance training, which was predictive of the strength of the aversive memory for the training experience (McIntyre et al., 2002). Furthermore both systemic and central activation of the adrenergic/noradrenergic system have been shown to enhance fear memory retention through activation of ß-adrenergic receptors in the BLA (McGaugh, 2015). Since peripheral adrenaline enters the brain poorly, and peripherally restricted ß-adrenergic antagonism has been shown to block the memory enhancing effects of adrenaline (Introini-Collison et al., 1992), it has been proposed that both peripheral and central ß-adrenergic receptor activation influence memory consolidation. Accordingly, our results show that either adrenal medullectomy or central blockade of noradrenergic transmission prevent the effects of ketamine. It is known that systemic adrenaline induces noradrenaline release within the brain (Gold and van Buskirk, 1978), likely by activation of ß-adrenergic receptors on the ascending vagus nerve that projects to brain stem noradrenergic nuclei, thus inducing noradrenaline release throughout the forebrain, including the BLA (McGaugh, 2015). In the light of this evidence, our results demonstrate that ketamine, administered immediately after a trauma, by increasing noradrenergic transmission peripherally and within the CNS, strongly potentiates memory consolidation for the traumatic event via activation of ß-adrenergic receptors in the BLA. Our finding that the memory enhancing effects were observed only when ketamine anesthesia was given immediately, but not 3-h, after training further confirms that the ketamine-induced noradrenergic potentiation underlies this mechanism. Indeed, it has been shown that soon after a stressful experience the BLA receives a strong noradrenergic input from the LC, which is relatively short lasting (less than 1-h) (Joëls et al., 2011). During this short time-window the BLA is concomitantly exposed to high levels of other stress hormones, neurotransmitters and neuromodulators which act in concert to enhance memory consolidation of the stressful event (Campolongo et al., 2009; Joëls et al., 2011; Morena et al., 2014). Intriguingly, our present results resemble

what we have previously shown with propofol anesthesia being able to enhance aversive memory consolidation only when given shortly after encoding, by interacting with endocannabinoids rapidly released after an inhibitory avoidance training (Hauer et al., 2011; Morena et al., 2014). Sleep has a profound effect on memory consolidation (Rasch and Born, 2013), therefore it might be argued that the effects of ketamine anesthesia on sleep might have indirectly influenced memory performance in our experiments. However, the lack of correlations between sleep onset or duration with retention latencies measured during the 48-h memory tests, allows us to exclude any potential contribution of sleeping property alterations induced by ketamine on its memory enhancing effects. Moreover, we did not find any significant difference when comparing sleep properties of anesthetized rats within all the experiments performed, thus showing that the different conditions (i.e. immediate vs delayed ketamine injection, adrenal medullectomy, LC lesion, systemic or intra-BLA injection of propranolol) had no influence on sleep onset and duration induced by ketamine. Previous studies examining the effects of ketamine on aversive memory processes have principally focused on the use of sub-anesthetic doses and have led to discrepant findings. In rats, pre-training injection of ketamine impaired acquisition of a cued fear conditioning paradigm (Pietersen et al., 2006). Conversely, other studies in the same species have found no effects of ketamine on acquisition or consolidation of cued fear conditioning (Bolton et al., 2012; Groeber Travis et al., 2015). Moreover, Goulart et al. (2010) reported that ketamine affected recognition memory in rats and Bolton et al. (2012) reported that ketamine impaired trace cued fear conditioning but had no effect of contextual fear conditioning. Two separate studies by Clifton et al. (2018) and McGowan et al. (2017) also reported no effects of sub-anesthetic ketamine administration on contextual fear memory consolidation (Clifton et al., 2018; McGowan et al., 2017). Interestingly, McGowan et al. (2017) reported that, when ketamine was given not in proximity of the trauma, but one week prior to contextual fear conditioning, it reduced freezing behavior at testing, thus showing a prophylactic effect (McGowan et al., 2017). Another study in mice has shown no effects of ketamine

on inhibitory avoidance memory retention (Wang et al., 2006). Ketamine is an N-methyl-d-aspartate (NMDA) receptors blocker and NMDA modulation has been shown to be crucially involved in memory modulation processes (Bliss and Collingridge, 1993; Izquierdo and Medina, 1997; Miserendino et al., 1990; Morris et al., 1986; Morris and Davis, 2012). Other NMDA receptor antagonists, such as MK-801, have been shown to affect memory in rodents when tested at subanesthetic doses or administered pre-training (Castellano et al., 1999; Roesler et al., 1999). Despite these evidences, data regarding the impairing effect of MK-801 on aversive memory are still inconclusive (Hsiung et al., 2020). Hsiung et al. (2020) have demonstrated that MK801 alters only the acquisition phase of memory but it enhances memory consolidation and memory retrieval of inhibitory avoidance training. MK-801-induced increases in shock sensitivity and locomotor activity in the pre-training regimen and the different doses used in the three studies could account at least in part for the confounding findings (Hsiung et al., 2020). Corroborating our previous (Morena et al., 2017) and present findings, one study showed that ketamine did increase fear memory retention also at sub-anesthetic doses when administered to rats in an active avoidance paradigm (Getova and Doncheva, 2011). Additionally, another study reported that repeated ketamine administration given after trauma exposure did not prevent PTSD symptomatology, and increased fear behavior to the trauma cue assessed a month later (Juven-Wetzler et al., 2014). Differences in doses, route and time of administration, animal species and strains used and behavioral paradigms might have accounted for these discrepancies. Conversely, except for few human findings reporting prophylactic effects of perioperative ketamine use for trauma-related disorders and depression (Ma et al., 2019; McGhee et al., 2008), there seems to be a general agreement in clinical studies showing that treatment with ketamine soon after accidental trauma is associated with increased symptoms of PTSD, such as dissociation, reexperiencing, hyperarousal and avoidance in the aftermath of the event (Schönenberg et al., 2008), which contribute to the development of a long-lasting PTSD symptomatology (Schönenberg et al., 2005) and are highly predictive of the severity and duration of subsequent PTSD (Bryant et

al., 2000). These reports corroborate our previous (Morena et al., 2017) and present findings that the use of ketamine anesthesia shortly after a traumatic event may represent a risk factor for the development of trauma-related disorders. Conversely, several evidence has reported that when ketamine is given in animal models for PTSD or in PTSD patients, not in proximity of the trauma or at sub-anesthetic doses, reduces PTSD symptomatology, including fear memory, anxiety- and depressive-like behavior in animals (Liriano et al., 2019; Zhang et al., 2015), while in humans it seems to be particularly effective in ameliorating comorbid depressive symptoms (Albott et al., 2018; Feder et al., 2014; Hartberg et al., 2018; Liriano et al., 2019; Womble, 2013). Therefore, there seems to be a dual, time-dependent effect of ketamine. When ketamine is given to patients suffering from PTSD it has beneficial effects, particularly in the presence of comorbid depression disorder, however, when given, as anesthetic, shortly after a traumatic event, in patients at risk of developing PTSD, it seems to facilitate and contribute to the development of a long-lasting PTSD symptomatology. Our present results strongly support this clinical evidence and indicate that the increased risk of PTSD development, associated with ketamine anesthesia, may arise from its memory consolidation enhancing properties. Furthermore, we also identify the possible neural underpinnings of the memory-enhancing effects of immediate post-trauma ketamine anesthesia by providing a candidate neuronal mechanism by which ketamine, through a strong, indirect peripheral and central noradrenergic activation, might potentiate traumatic memory consolidation, and contribute to the development of PTSD later in life. Accordingly, in the clinical setting the combination of ketamine anesthesia with dexmedetomidine, which decreases noradrenergic activation, has been shown to reduce both the sympathomimetic effects, including increased cardiovascular response, and postoperative psychiatric adverse reaction induced by ketamine (Levanen et al., 1995). Given that PTSD is a highly debilitating psychiatric disorder and very difficult to treat, our findings present a strong translational value, as they have the potential to inform clinicians for a better management of ketamine anesthesia in emergency care and trauma victims. For instance, coupling ketamine injection with ß-blockers or α_2-

adrenoceptor agonists, such as dexmedetomidine, which have already been shown to reduce traumatic memory consolidation and PTSD symptomatology (Giustino et al., 2016; Morena et al., 2017; Pandharipande et al., 2007), would potentially have a prophylactic effect and help to prevent the occurrence and development of trauma-related disorders later in life.

Conclusions

Here we identified a possible mechanism for the memory-enhancing effects induced by ketamine anesthesia when administered immediately after a traumatic event. Particularly, our results indicate that the memory enhancing effects of ketamine anesthesia are time-dependent, as it enhances memory only when given in close proximity of the trauma. Furthermore, our findings indicate that these memory effects are mediated by a combined peripheral-central potentiation of the sympathetic nervous system induced by ketamine. Taken together, our findings lay the basis for future clinical studies to potentially guide clinicians for a better management of sedation/anesthesia in emergency care and reduce the risk of developing trauma-related disorders. in trauma victims.

References

Albott, C. S., Lim, K. O., Forbes, M. K., Erbes, C., Tye, S. J., Grabowski, J. G., … Shiroma, P. R. (2018). Efficacy, Safety, and Durability of Repeated Ketamine Infusions for Comorbid Posttraumatic Stress Disorder and Treatment-Resistant Depression. The Journal of Clinical Psychiatry, 79(3), pii: 17m11634. https://doi.org/10.4088/JCP.17m11634

American Psychiatric Association. (2013). Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association. https://doi.org/10.1176/appi.books.9780890425596

Asan, E. (1998). The catecholaminergic innervation of the rat amygdala. Advances in Anatomy, Embryology, and Cell Biology, 142, 1–118. https://doi.org/10.1007/978-3-642-72085-7

Atsak, P., Hauer, D., Campolongo, P., Schelling, G., Fornari, R. V., & Roozendaal, B. (2015). Endocannabinoid Signaling within the Basolateral Amygdala Integrates Multiple Stress Hormone Effects on Memory Consolidation. Neuropsychopharmacology, 40(6), 1485–1494. https://doi.org/10.1038/npp.2014.334

Baraka, A., Harrison, T., & Kachachi, T. (1973). Catecholamine levels after ketamine anesthesia in man. Anesthesia and Analgesia, 52(2), 198–200.

Bliss, T. V. P., & Collingridge, G. L. (1993). A synaptic model of memory: Long-term potentiation in the hippocampus. Nature. https://doi.org/10.1038/361031a0

Bolton, M. M., Heaney, C. F., Sabbagh, J. J., Murtishaw, A. S., Magcalas, C. M., & Kinney, J. W. (2012). Deficits in emotional learning and memory in an animal model of schizophrenia. Behavioural Brain Research, 233(1), 35–44. https://doi.org/10.1016/j.bbr.2012.04.049

Bryant, R. A., Moulds, M. L., & Guthrie, R. M. (2000). Acute Stress Disorder Scale: a self-report measure of acute stress disorder. Psychological Assessment, 12(1), 61–68.

Campolongo, P., Roozendaal, B., Trezza, V., Cuomo, V., Astarita, G., Fu, J., … Piomelli, D. (2009). Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. Proceedings of the National Academy of Sciences of the United States of America, 106(19), 8027–8031. https://doi.org/10.1073/pnas.0903038106

Campolongo, P., Roozendaal, B., Trezza, V., Hauer, D., Schelling, G., McGaugh, J. L., & Cuomo, V. (2009). Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. Proceedings of the National Academy of Sciences of the United States of America, 106(12), 4888–4893. https://doi.org/10.1073/pnas.0900835106

Castellano, C., Cestari, V., Ciamei, A., & Pavone, F. (1999). MK-801-induced disruptions of one-trial inhibitory avoidance are potentiated by stress and reversed by naltrexone. Neurobiology of Learning and Memory, 72(3), 215–229. https://doi.org/10.1006/nlme.1999.3908

Clifton, N. E., Thomas, K. L., & Hall, J. (2018). The effect of ketamine on the consolidation and extinction of contextual fear memory. Journal of Psychopharmacology (Oxford, England), 32(2), 156–162. https://doi.org/10.1177/0269881117748903

El Iskandrani, K. S., Oosterhof, C. A., El Mansari, M., & Blier, P. (2015). Impact of subanesthetic doses of ketamine on AMPA-mediated responses in rats: An in vivo electrophysiological study on monoaminergic and glutamatergic neurons. Journal of Psychopharmacology (Oxford, England), 29(7), 792–801. https://doi.org/10.1177/0269881115573809

Feder, A., Parides, M. K., Murrough, J. W., Perez, A. M., Morgan, J. E., Saxena, S., … Charney, D. S. (2014). Efficacy of intravenous ketamine for treatment of chronic posttraumatic stress disorder: A randomized clinical trial. JAMA Psychiatry, 71(6), 681–688. https://doi.org/10.1001/jamapsychiatry.2014.62

Ferrari, L. F., Gear, R. W., & Levine, J. D. (2010). Attenuation of activity in an endogenous analgesia circuit by ongoing pain in the rat. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 30(41), 13699–13706. https://doi.org/10.1523/JNEUROSCI.2867-10.2010

Getova, D. P., & Doncheva, N. D. (2011). Effects of ketamine on memory and nociception in rats. Folia Medica, 53(1), 53–59. https://doi.org/10.2478/v10153-010-0028-8

Giustino, T. F., Fitzgerald, P. J., & Maren, S. (2016, April). Revisiting propranolol and PTSD: Memory erasure or extinction enhancement? Neurobiology of Learning and Memory. Academic Press Inc. https://doi.org/10.1016/j.nlm.2016.01.009

Gold, P. E., & van Buskirk, R. (1978). Posttraining brain norepinephrine concentrations: Correlation with retention performance of avoidance training and with peripheral epinephrine modulation of memory processing. Behavioral Biology, 23(4), 509–520. https://doi.org/10.1016/S0091-6773(78)91614-0

Goulart, B. K., de Lima, M. N. M., de Farias, C. B., Reolon, G. K., Almeida, V. R., Quevedo, J., … Roesler, R. (2010). Ketamine impairs recognition memory consolidation and prevents learning-induced increase in hippocampal brain-derived neurotrophic factor levels. Neuroscience, 167(4), 969–973. https://doi.org/10.1016/j.neuroscience.2010.03.032

Green, S. M., Rothrock, S. G., Harris, T., Hopkins, G. A., Garrett, W., & Sherwin, T. (1998). Intravenous ketamine for pediatric sedation in the Emergency Department: Safety profile with 156 cases. Academic Emergency Medicine, 5(10), 971–976. https://doi.org/10.1111/j.1553-2712.1998.tb02773.x

Groeber Travis, C. M., Altman, D. E., & Genovese, R. F. (2015). Ketamine administration diminishes operant responding but does not impair conditioned fear. Pharmacology, Biochemistry, and Behavior, 139(Pt A), 84–91. https://doi.org/10.1016/j.pbb.2015.10.013

Guo, T. Z., Jiang, J. Y., Buttermann, A. E., & Maze, M. (1996). Dexmedetomidine injection into the locus ceruleus produces antinociception. Anesthesiology, 84(4), 873–881. https://doi.org/10.1097/00000542-199604000-00015

Hara, K., Yanagihara, N., Minami, K., Ueno, S., Toyohira, Y., Sata, T., … Izumi, F. (1998). Ketamine interacts with the noradrenaline transporter at a site partly overlapping the desipramine binding site. Naunyn-Schmiedeberg's Archives of Pharmacology, 358(3), 328–333. https://doi.org/10.1007/PL00005261

Harrison, N. L., & Simmonds, M. A. (1985). Quantitative studies on some antagonists of N-methyl D-aspartate in slices of rat cerebral cortex. British Journal of Pharmacology, 84(2), 381–391. https://doi.org/10.1111/j.1476-5381.1985.tb12922.x

Hartberg, J., Garrett-Walcott, S., & De Gioannis, A. (2018). Impact of oral ketamine augmentation on hospital admissions in treatment-resistant depression and PTSD: a retrospective study. Psychopharmacology, 235(2), 393–398. https://doi.org/10.1007/s00213-017-4786-3

Hauer, D., Ratano, P., Morena, M., Scaccianoce, S., Briegel, I., Palmery, M., … Campolongo, P. (2011). Propofol enhances memory formation via an interaction with the endocannabinoid system. Anesthesiology, 114(6), 1380–1388. https://doi.org/10.1097/ALN.0b013e31821c120e

Hsiung, M. H., Huang, W. L., Kan, L. Y., Chen, L. H., & Hu, S. S. J. (2020). The facilitating effect of MK-801 on inhibitory avoidance memory via mTOR signaling in the mouse hippocampus. Behavioural Brain Research, 389. https://doi.org/10.1016/j.bbr.2020.112630

Introini-Collison, I., Saghafi, D., Novack, G. D., & McGaugh, J. L. (1992). Memory-enhancing effects of post-training dipivefrin and epinephrine: Involvement of peripheral and central adrenergic receptors. Brain Research, 572(1–2), 81–86. https://doi.org/10.1016/0006-8993(92)90454-H

Izquierdo, I., & Medina, J. H. (1997). Memory formation: The sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiology of Learning and Memory, 68(3), 285–316. https://doi.org/10.1006/nlme.1997.3799

Joëls, M., Fernandez, G., & Roozendaal, B. (2011, June). Stress and emotional memory: A matter of timing. Trends in Cognitive Sciences. https://doi.org/10.1016/j.tics.2011.04.004

Juven-Wetzler, A., Cohen, H., Kaplan, Z., Kohen, A., Porat, O., & Zohar, J. (2014). Immediate ketamine treatment does not prevent posttraumatic stress responses in an animal model for PTSD. European Neuropsychopharmacology, 24(3), 469–479. https://doi.org/10.1016/j.euroneuro.2013.08.007

Khasar, S. G., Miao, F. J. P., Jänig, W., & Levine, J. D. (1998). Vagotomy-induced enhancement of mechanical hyperalgesia in the rat is sympathoadrenal-mediated. Journal of Neuroscience, 18(8), 3043–3049. https://doi.org/10.1523/jneurosci.18-08-03043.1998

Lashgari, R., Khakpour-Taleghani, B., Motamedi, F., & Shahidi, S. (2008). Effects of reversible inactivation of locus coeruleus on long-term potentiation in perforant path-DG synapses in rats. Neurobiology of Learning and Memory, 90(2), 309–316. https://doi.org/10.1016/j.nlm.2008.05.012

Levanen, J., Makela, M. L., & Scheinin, H. (1995). Dexmedetomidine premedication attenuates ketamine-induced cardiostimulatory effects and postanesthetic delirium. Anesthesiology, 82(5), 1117–1125. https://doi.org/10.1097/00000542-199505000-00005

Liriano, F., Hatten, C., & Schwartz, T. L. (2019). Ketamine as treatment for post-traumatic stress disorder: A review. Drugs in Context. Bioexcel Publishing LTD. https://doi.org/10.7573/dic.212305

Ma, J. H., Wang, S. Y., Yu, H. Y., Li, D. Y., Luo, S. C., Zheng, S. S., … Duan, K. M. (2019). Prophylactic use of ketamine reduces postpartum depression in Chinese women undergoing cesarean section☆. Psychiatry Research, 279, 252–258. https://doi.org/10.1016/j.psychres.2019.03.026

McGaugh, J. L. (2000, January). Memory - A century of consolidation. Science. American Association for the Advancement of Science. https://doi.org/10.1126/science.287.5451.248

McGaugh, J. L. (2015). Consolidating Memories. Annual Review of Psychology, 66(1), 1–24. https://doi.org/10.1146/annurev-psych-010814-014954

McGaugh, J. L., & Dawson, R. G. (1971). Modification of memory storage processes. Behavioral Science, 16(1), 45–63. https://doi.org/10.1002/bs.3830160105

McGhee, L. L., Maani, C. V., Garza, T. H., Gaylord, K. M., & Black, I. H. (2008). The correlation between ketamine and posttraumatic stress disorder in burned service members. The Journal of Trauma, 64(2 Suppl). https://doi.org/10.1097/ta.0b013e318160ba1d

McGowan, J. C., LaGamma, C. T., Lim, S. C., Tsitsiklis, M., Neria, Y., Brachman, R. A., & Denny, C. A. (2017). Prophylactic Ketamine Attenuates Learned Fear. Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology, 42(8), 1577–1589. https://doi.org/10.1038/npp.2017.19

McIntyre, C. K., Hatfield, T., & McGaugh, J. L. (2002). Amygdala norepinephrine levels after training predict inhibitory avoidance retention performance in rats. European Journal of Neuroscience, 16(7), 1223–1226. https://doi.org/10.1046/j.1460-9568.2002.02188.x

Miserendino, M. J. D., Sananes, C. B., Melia, K. R., & Davis, M. (1990). Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala. Nature, 345(6277), 716–718. https://doi.org/10.1038/345716a0

Morena, M., Aukema, R. J., Leitl, K. D., Rashid, A. J., Vecchiarelli, H. A., Josselyn, S. A., & Hill, M. N. (2019). Upregulation of Anandamide Hydrolysis in the Basolateral Complex of Amygdala Reduces Fear Memory Expression and Indices of Stress and Anxiety. The Journal of

Neuroscience: The Official Journal of the Society for Neuroscience, 39(7), 1275–1292. https://doi.org/10.1523/jneurosci.2251-18.2018

Morena, M., Berardi, A., Peloso, A., Valeri, D., Palmery, M., Trezza, V., … Campolongo, P. (2017). Effects of ketamine, dexmedetomidine and propofol anesthesia on emotional memory consolidation in rats: Consequences for the development of post-traumatic stress disorder. Behavioural Brain Research, 329, 215–220. https://doi.org/10.1016/j.bbr.2017.04.048

Morena, M., Leitl, K. D., Vecchiarelli, H. A., Gray, J. M., Campolongo, P., & Hill, M. N. (2016). Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety. Neuropharmacology, 111, 59–69. https://doi.org/10.1016/j.neuropharm.2016.08.020

Morena, M., Roozendaal, B., Trezza, V., Ratano, P., Peloso, A., Hauer, D., … Campolongo, P. (2014). Endogenous cannabinoid release within prefrontal-limbic pathways affects memory consolidation of emotional training. Proceedings of the National Academy of Sciences, 111(51), 18333–18338. https://doi.org/10.1073/pnas.1420285111

Morris, R. G. M., Anderson, E., Lynch, G. S., & Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature, 319(6056), 774–776. https://doi.org/10.1038/319774a0

Morris, R. G. M., & Davis, M. (2012). The role of NMDA receptors in learning and memory. In The NMDA Receptor. https://doi.org/10.1093/acprof:oso/9780192625021.003.0015

Mouton, P. R., Pakkenberg, B., Gundersen, H. J., & Price, D. L. (1994). Absolute number and size of pigmented locus coeruleus neurons in young and aged individuals. Journal of Chemical Neuroanatomy, 7(3), 185–190. https://doi.org/10.1016/0891-0618(94)90028-0

Nishimura, M., Sato, K., Okada, T., Yoshiya, I., Schloss, P., Shimada, S., & Tohyama, M. (1998). Ketamine inhibits monoamine transporters expressed in human embryonic kidney 293 cells. Anesthesiology, 88(3), 768–774. https://doi.org/10.1097/00000542-199803000-00029

Pandharipande, P. P., Pun, B. T., Herr, D. L., Maze, M., Girard, T. D., Miller, R. R., … Ely, E. W. (2007). Effect of sedation with dexmedetomidine vs lorazepam on acute brain dysfunction in mechanically ventilated patients: MENDS (dex v loraz sedation). JAMA: The Journal of the American Medical Association, 298(22), 2644–2653. https://doi.org/10.1001/jama.298.22.2644

Pietersen, C. Y., Bosker, F. J., Postema, F., Fokkema, D. S., Korf, J., & den Boer, J. A. (2006). Ketamine administration disturbs behavioural and distributed neural correlates of fear conditioning in the rat. Progress in Neuro-Psychopharmacology & Biological Psychiatry, 30(7), 1209–1218. https://doi.org/10.1016/j.pnpbp.2006.02.019

Pitman, R. K., Rasmusson, A. M., Koenen, K. C., Shin, L. M., Orr, S. P., Gilbertson, M. W., … Liberzon, I. (2012, November). Biological studies of post-traumatic stress disorder. Nature Reviews Neuroscience. https://doi.org/10.1038/nrn3339

Quirarte, G. L., Roozendaal, B., & Mcgaugh, J. L. (1997). Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. Proceedings of the National Academy of Sciences of the United States of America, 94(25), 14048–14053. https://doi.org/10.1073/pnas.94.25.14048

Rasch, B., & Born, J. (2013). About sleep's role in memory. Physiological Reviews, 93(2), 681–766. https://doi.org/10.1152/physrev.00032.2012

Ratano, P., Petrella, C., Forti, F., Passeri, P. P., Morena, M., Palmery, M., … Campolongo, P.

(2018). Pharmacological inhibition of 2-arachidonoilglycerol hydrolysis enhances memory consolidation in rats through CB2 receptor activation and mTOR signaling modulation. Neuropharmacology, 138, 210–218. https://doi.org/10.1016/j.neuropharm.2018.05.030

Read, M. D., Maani, C. V., & Blackwell, S. (2017). Dexmedetomidine as a Rescue Therapy for Emergence Delirium in Adults: A Case Series. A & A Case Reports, 9(1), 20–23. https://doi.org/10.1213/XAA.0000000000000510

Roesler, R., Vianna, M. R. M., De-Paris, F., & Quevedo, J. (1999). Memory-enhancing treatments do not reverse the impairment of inhibitory avoidance retention induced by NMDA receptor blockade. Neurobiology of Learning and Memory, 72(3), 252–258. https://doi.org/10.1006/nlme.1999.3910

Roozendaal, B., Hui, G. K., Hui, I. R., Berlau, D. J., McGaugh, J. L., & Weinberger, N. M. (2006). Basolateral amygdala noradrenergic activity mediates corticosterone-induced enhancement of auditory fear conditioning. Neurobiology of Learning and Memory, 86(3), 249–255. https://doi.org/10.1016/j.nlm.2006.03.003

Roozendaal, B., McReynolds, J. R., & McGaugh, J. L. (2004). The Basolateral Amygdala Interacts with the Medial Prefrontal Cortex in Regulating Glucocorticoid Effects on Working Memory Impairment. Journal of Neuroscience, 24(6), 1385–1392. https://doi.org/10.1523/JNEUROSCI.4664-03.2004

Schönenberg, M., Reichwald, U., Domes, G., Badke, A., & Hautzinger, M. (2005). Effects of peritraumatic ketamine medication on early and sustained posttraumatic stress symptoms in moderately injured accident victims. Psychopharmacology, 182(3), 420–425. https://doi.org/10.1007/s00213-005-0094-4

Schönenberg, M., Reichwald, U., Domes, G., Badke, A., & Hautzinger, M. (2008). Ketamine aggravates symptoms of acute stress disorder in a naturalistic sample of accident victims. Journal of Psychopharmacology, 22(5), 493–497. https://doi.org/10.1177/0269881107082481

Shehabi, Y., Grant, P., Wolfenden, H., Hammond, N., Bass, F., Campbell, M., & Chen, J. (2009). Prevalence of delirium with dexmedetomidine compared with morphine based therapy after cardiac surgery: A randomized controlled trial (DEXmedetomidine compared to morphine-DEXCOM study). Anesthesiology, 111(5), 1075–1084. https://doi.org/10.1097/ALN.0b013e3181b6a783

Sleigh, J., Harvey, M., Voss, L., & Denny, B. (2014). Ketamine - more mechanisms of action than just NMDA blockade. Trends in Anaesthesia and Critical Care. Churchill Livingstone. https://doi.org/10.1016/j.tacc.2014.03.002

Song, Z., Ansah, O. B., Meyerson, B. A., Pertovaara, A., & Linderoth, B. (2013). Exploration of supraspinal mechanisms in effects of spinal cord stimulation: Role of the locus coeruleus. Neuroscience, 253, 426–434. https://doi.org/10.1016/j.neuroscience.2013.09.006

Wang, J. H., Fu, Y., Wilson, F. A. W., & Ma, Y. Y. (2006). Ketamine affects memory consolidation: differential effects in T-maze and passive avoidance paradigms in mice. Neuroscience, 140(3), 993–1002. https://doi.org/10.1016/j.neuroscience.2006.02.062

Weant, K. A., Davis, W. D., Davis, K. A., & Hooper, K. (2019). The Use of Ketamine for the Management of Acute Pain in the Emergency Department. Advanced Emergency Nursing Journal, 41(2), 111–121. https://doi.org/10.1097/TME.0000000000000238

White, P. F., Way, W. L., & Trevor, A. J. (1982). Ketamine - Its pharmacology and therapeutic uses. Anesthesiology, 56(2), 119–136. https://doi.org/10.1097/00000542-198202000-00007

Wilkinson, C. W., Shinsako, J., & Dallman, M. F. (1981). Return of pituitary-adrenal function after adrenal enucleation or transplantation: Diurnal rhythms and responses to ether. Endocrinology, 109(1), 162–169. https://doi.org/10.1210/endo-109-1-162

Winter, H., & Irle, E. (2004). Hippocampal volume in adult burn patients with and without posttraumatic stress disorder. The American Journal of Psychiatry, 161(12), 2194–2200. https://doi.org/10.1176/appi.ajp.161.12.2194

Womble, A. L. (2013). Effects of ketamine on major depressive disorder in a patient with posttraumatic stress disorder. AANA Journal, 81(2), 118–119.

Wotjak, C. T. (2019). Sound check, stage design and screen plot - how to increase the comparability of fear conditioning and fear extinction experiments. Psychopharmacology, 236(1), 33–48. https://doi.org/10.1007/s00213-018-5111-5

Zanos, P., Moaddel, R., Morris, P. J., Georgiou, P., Fischell, J., Elmer, G. I., … Gould, T. D. (2016). NMDAR inhibition-independent antidepressant actions of ketamine metabolites. Nature, 533(7604), 481–486. https://doi.org/10.1038/nature17998

Zhang, L. M., Zhou, W. W., Ji, Y. J., Li, Y., Zhao, N., Chen, H. X., … Li, Y. F. (2015). Anxiolytic effects of ketamine in animal models of posttraumatic stress disorder. Psychopharmacology, 232(4), 663–672. https://doi.org/10.1007/s00213-014-3697-9

Zhao, Y., & Sun, L. (2008). Antidepressants modulate the in vitro inhibitory effects of propofol and ketamine on norepinephrine and serotonin transporter function. Journal of Clinical Neuroscience, 15(11), 1264–1269. https://doi.org/10.1016/j.jocn.2007.11.007

CHAPTER 3

Sex-divergent long-term effects of single prolonged stress in adult rats

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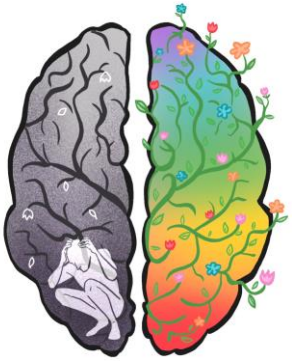


Abstract

Single prolonged stress (SPS) is an experimental model that recapitulates in rodents some of the core symptoms of post-traumatic stress disorder (PTSD). Although women have a two-fold greater risk to develop PTSD, most preclinical studies have been carried out in males. Furthermore, the long-term persistence of behavioral alterations induced by SPS has been rarely investigated. Here, we evaluated the long-term effects of SPS on PTSD-relevant behavioral domains in rats and whether these effects were sex-dependent. To this aim, separate cohorts of male and female adult rats were subjected to SPS and, 30 days later, long-term effects were assessed. We found that SPS exposure reduced locomotor activity in both sexes in an open field task. Males only showed increased anxiety-like behavior in the elevated plus maze and marble burying tests, enhanced acoustic startle response and impaired spatial memory retention while females were unaffected. SPS exposure did not alter auditory fear memory dynamics in males, but it did alter extinction retrieval in females. We provide the first evidence that SPS reproduces long-term emotional alterations in male, but not in female, rats which persist 30 days following trauma exposure, thus resembling some of the hallmark symptoms of PTSD. Furthermore, our results show for the first time a long-term SPS-induced alteration of cued fear extinction in females. Our findings are relevant to future research on trauma-related disorders and may help develop sex-specific interventions to treat PTSD.

Introduction

Exposure to a traumatic event may enhance the risk to develop psychiatric diseases, such as post-traumatic stress disorder (PTSD), which is characterized by hyperarousal, hypervigilance, increased anxiety, altered sociability and cognitive alterations (e.g. over-consolidation, memory generalization and impaired extinction of the trauma) [1,2]. Even if the vast majority of individuals will experience a trauma once throughout their life [3], only a small subset of them develops PTSD, among those, women present a two-fold higher risk for developing this disorder [4–6]. Since the ability to examine the neurobiological underpinnings of PTSD in humans is limited, preclinical models represent a valuable tool to explore the long-term consequences of trauma exposure [7–10]. Single prolonged stress (SPS) paradigm is a valid model to resemble the core symptoms of PTSD [11]. It has been described for the first time by Liberzon et al. over 20 years ago as a multimodal stress protocol including sequential exposure to 2 h of restraint stress (psychological stimulus), 20 min of forced swim stress (physical stimulus) and inhalation of gas anesthetic until loss of consciousness (pharmacological stimulus) [12,13]. Growing evidence has demonstrated that SPS profoundly affects behavior in rodents resembling the hallmark symptoms observed in PTSD patients [13,14], including enhanced anxiety and hyperarousal [15–18], fear memory dysfunctions [19–22], alterations in social interaction and sleep [23,24]. The manifestation of a PTSD-like phenotype following SPS exposure is time-dependent and requires 7-14 days of incubation (i.e. sensitization or consolidation period) to develop [22,25,26]. Thus, in most of the studies investigating the neurobiology of PTSD, rodents subjected to a SPS protocol are left undisturbed for this time window and tested 7-14 days after trauma exposure [11,25]. Given that PTSD is a chronic psychiatric disease, it is of critical importance to evaluate whether the effects induced by SPS persist long after stress exposure [27]. However, only few studies investigated the long-term alterations induced by SPS reporting conflicting results [28,29]. As mentioned before, women are more susceptible to develop PTSD compared to men

[4–6]. Furthermore, men and women are exposed to different types of traumatic events, for instance women are more exposed to sexual violence and men are more predisposed to accidents and physical violence than women [30]. Importantly, women have been reported to develop different symptoms and a more chronic course of PTSD with respect to men [5,31]. However, the neurobiological mechanisms underlying these gender differences remain unclear due to the paucity of both clinical and preclinical studies carried out in female subjects [32,33]. Moreover, studies comparing both sexes are still inconsistent and have led to controversial results. While hyper-responsiveness to stressful events and alterations in fear memory processes were found in men affected by PTSD and in SPS-exposed male rodents as well [7,11,13,14], the effects in females are less clear. Studies have demonstrated that women with PTSD do not display the same stress-responsiveness as men [34]. Further, the few preclinical studies examining sex differences in SPS-induced emotional and cognitive behavioral dysfunctions have reported conflicting results in female rats: no effects on anxiety-like behavior [35,36], increased anxiety [37], no cognitive alterations [38] or impaired extinction and enhanced fear retrieval [39]. Moreover, in the above-mentioned studies SPS-induced behavioral alterations have been evaluated following only 1 week of incubation, a relatively short period of time to resemble the chronicity nature of PTSD. Thus, the present study examined whether SPS induced persistent PTSD-like behavioral alterations in rats long after trauma exposure and whether these effects are sex-dependent. To this aim, male and female adult rats were subjected to a single session of SPS and behavioral outcomes were assessed 30 days later in a battery of tests. Specifically, we evaluated locomotor activity in an open field task, anxiety-like behavior, avoidance and hyperarousal in an elevated plus maze, marble burying and acoustic startle tasks. Spatial and cued fear memory dynamics were assessed in a Morris water maze and an auditory fear conditioning paradigm, respectively.

Materials and methods

Animals

Male and female adult Sprague–Dawley rats (350-400 g and 250-300 g at the time of the behavioral experiments, respectively) from Charles River Laboratories (Calco, Italy) were pair housed in a temperature controlled (21 ± 1°C) vivarium room and maintained under a 12 h/12 h light/dark cycle (7:00 A.M. to 7:00 P.M. lights on). Food and water were available ad libitum. All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, the D. L. 26/2014 and were approved by the Italian Ministry of Health.

Experimental design

As shown in Fig.1, separate cohorts of adult male and female rats at post-natal day (PND) 90 were randomly assigned to the trauma-exposed (SPS-male and SPS-female groups) or control groups (CTRL-male and CTRL-female groups) and were subjected to a SPS procedure. After being exposed to SPS, rats were divided into three separate groups and underwent a behavioral test battery 30 days later. The first group was exposed to the open field (PND 120) and two days later (PND 122) to the Morris water maze tasks, the second group was exposed to the elevated plus maze (PND 120) and to the acoustic startle response (PND 122) tasks and the third group was exposed to the marble burying (PND 120) and to the auditory fear conditioning (PND 122) tasks. One animal of the SPS-female group fell off the elevated plus maze apparatus was excluded from statistical analyses.

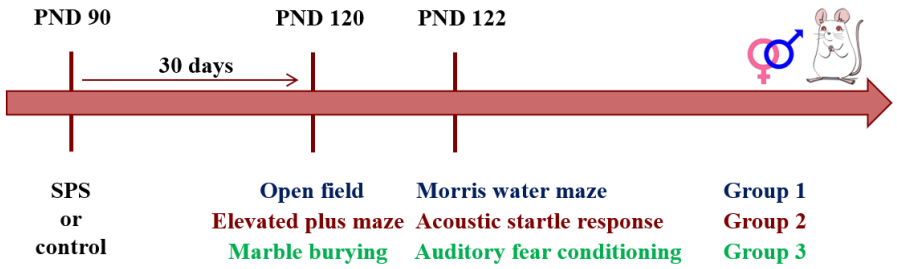


Figure 1: Experimental timeline. Separate cohorts of adult male and female rats at post-natal day (PND) 90 were randomly assigned to the trauma-exposed (SPS-male and SPS-female groups) or control groups (CTRL-male and CTRL-female groups) and were subjected to a SPS procedure. To assess long-term effects of SPS on locomotor activity, emotional and cognitive alterations, 30 days after stress exposure, rats were divided into three separate groups and underwent a behavioral test battery. The first group was exposed to the open field (PND 120) and two days later (PND 122) to the Morris water maze tasks, the second group was exposed to the elevated plus maze (PND 120) and to the acoustic startle response (PND 122) tasks and the third group was exposed to the marble burying (PND 120) and to the auditory fear conditioning (PND 122) tasks.

Single prolonged stress protocol

We used a slightly modified SPS protocol as the ones previously described [12,40]. Briefly, separate groups of male and female rats at PND 90 were exposed to a single session of SPS which consisted of 2 hours of restraint stress in a restrainer (30 cm in length with an inside diameter of 7.5 cm), 15 min of forced swim stress (24±1 °C water temperature) followed by 15 min of recovery, and then isoflurane exposure until loss of consciousness (4% for induction and 1.5–2.5% for maintenance, in oxygen). Rats not assigned to SPS groups were placed in a separate room throughout the duration of SPS. After SPS procedure, all rats were returned to their home cage and left undisturbed for 30 days.

Behavioral tests

Open field

In the open field test each rat was placed into the corner of the open field arena (80 × 80 × 60 cm). The test was performed under dim light conditions (2 lx) and lasted 20 min, as previously described [41]. The total distance traveled (cm) by each rat was determined as an index of the rat locomotor activity. After each session, rats were returned to their home cage, fecal boli were removed and the walls and floor of the arena were cleaned with a 70% ethanol solution. The total distance traveled was acquired and analyzed using an automated video-tracking system (Smart, Panlab, Barcelona, Spain).

Elevated plus maze

The test was performed as previously described [42,43]. The elevated plus maze apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) that extended from a common central platform (10 × 10 cm). The apparatus, made of plexiglass (black floor and walls), was elevated to a height of 60 cm above the floor level. Rat's behavior was recorded for 5 min by using a video camera positioned above the experimental apparatus and videos were analyzed with Observer XT 12 (Noldus Information Technology BV, Wageningen, The Netherlands) by observers blinded to the experimental conditions. Each rat was individually placed on the central platform facing a closed arm. Time spent in the open arms (s) and number of entries in the open arms were analyzed. After each session, rats were returned to their home cage, fecal boli were removed and the apparatus was cleaned with a 70% ethanol solution.

Acoustic startle response

A slightly modified procedure as the one previously described [40] was used. Rats

were placed in a startle reflex apparatus (Med Associates, Fairfax, Vermont) for a 5 min acclimatization period with a 70 dB background noise, which continued during all the session. Each session consisted of 10 pulse trials (115 dB) with intertrial intervals selected randomly between 10 and 15 s. Acoustic devices and startle cages were connected to a computer, which detected and analyzed all chamber variables using customized software (Med Associates, Fairfax, Vermont). The system allows recording and analysis of the signal generated by the animal movement through a high sensitivity weight transducer system. The maximal startle reflex response for each animal was calculated as the average of the responses to the 10 pulse trials.

Marble burying

We used a slightly modified protocol as the one previously described [44]. The marble burying test was conducted in a quadrangular arena (40 × 40 × 60 cm) made of plexiglass with clean sawdust covering the floor, under dim light condition. Twenty-five standard glass marbles (1.5 cm diameter, arranged in five rows of five marbles each) were placed uniformly over the surface. Individual rats were placed in the marble arena and activity was monitored for 30 min by a video camera placed above the arena. At the end of the session, animals were gently removed from the arena and returned to their home cage, and the number of marbles buried was counted. New bedding was used for each animal, and marbles were cleaned with 70 % ethanol solution.

Morris water maze

The experimental apparatus was a circular tank (1.83 m in diameter and 0.58 m in height) filled with water (23–24 °C) to a depth of 20 cm. The maze was located in a room containing many salient, visual, extra-maze cues. During the spatial training a rectangular platform (20 x 25 cm) was placed at a fixed location 25 cm away from the

edge of the pool and 2.5 cm below the water surface. The experiments were performed according to the procedure previously described [45,46]. For spatial training, the rats were given four trials on each daily session for three consecutive days. On each trial, the animal was placed in the tank facing the wall at one of the 4 designated start positions and allowed to escape onto the hidden platform. If an animal failed to find the platform within 60 s during the first day of the training, it was manually guided to the platform. The rat was allowed to remain on the platform for 10 s and was then placed into a holding cage for 25 s until the start of the next trial. The time each animal spent to reach the platform was recorded as the escape latency. Retention of the spatial training was assessed 24 h after the last training session with a 60 s free-swim probe trial and a new starting position was used. Training and probe trials were videotaped, and an automated tracking system (Smart, Panlab, Barcelona, Spain) was used to analyze the swim path of each subject and calculate the initial latency to cross the platform location and the number of crossings through the platform location. The target and opposite quadrants were equidistant from the starting position on the probe trial.

Auditory fear conditioning

We used a slightly modified protocol as previously described [47,48]. All the phases of the auditory fear conditioning task were performed in the same operant chambers, located in sound-attenuating cubicles (Med Associates, Fairfax, Vermont). The floor of the chambers consisted of stainless-steel bars that delivered a scrambled electric footshock. On day 1, rats received 5 habituation tones (30 s, 4 kHz, 75 dB; 3 min intertrial interval), immediately followed by 7 conditioning tones that co-terminated with footshocks (0.5 s, 0.65 mA). On day 2, rats were returned to the chambers for the extinction session, which consisted of 12 tones in the absence of footshock. On day 3, rats were returned to the chambers and presented with 8 tones in the absence of footshock to test for extinction retrieval. Freezing behavior, defined as the absence of

all movements except for those related to breathing [49], served as the measurement of fear and was monitored with digital video cameras. An experimenter blinded to the experimental conditions quantified the total time that rats spent freezing during the 30 s tone presentations by using a digital stopwatch. The percentage of time spent in freezing during the 30 s tone presentations was calculated as: [time spent in freezing during the tone presentation (s) / 30 s] x 100. For the conditioning session (day 1), the time spent in freezing during the first and the last tone presentation was represented, while for day 2 and 3 the average of percentage of time spent in freezing during all trials was represented.

Statistical analysis

Statistical analysis was performed using Statiew statistical software. Data are expressed as mean ± SEM. Escape latencies during the acquisition phase of the Morris water maze and the percentage of freezing during the conditioning phase of the auditory fear conditioning were analyzed with a repeated measures ANOVA (RM ANOVA). In all the other cases, data were analyzed with two-way ANOVA. Tukey-Kramer post-hoc tests were performed to control for significant differences between groups when appropriate. A probability level of < 0.05 was accepted as statistically significant.

Results

Exposure to SPS induced a long-term decrease of locomotor activity in male and female rats

Long-term effects of SPS on locomotor activity were assessed in male and female adult rats 30 days after stress exposure. As shown in Fig.2a, two-way ANOVA for the distance traveled in the open field revealed significant effects of SPS ($F_{(1,35)} = 12.816$; $P = 0.001$) and sex ($F_{(1,35)} = 130.675$; $P < 0.0001$), but no interaction between these

two factors ($F_{(1,35)}$ = 0.046; P = 0.831). These results indicate that exposure to SPS reduced locomotion in both sexes. The representative path tracks indicating the locomotor activity in the open field arena of all groups of rats are shown in Fig.2b.

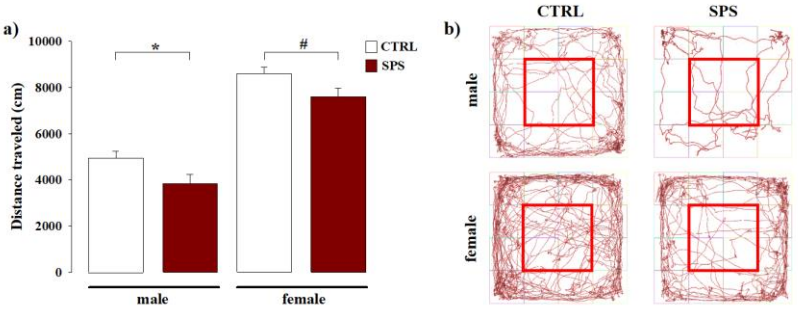


Figure 2: Long-term effects of SPS on locomotor activity in the open field task. SPS exposure reduced locomotion in both sexes, 30 days after trauma in the open field task. (a) Histograms show the effects of SPS on the total distance traveled (cm) in the open field task and (b) representative path tracks of 1 animal per group indicating the exploratory activity in the open field arena. *, P < 0.05, vs. CTRL-male group; #, P < 0.05 vs. CTRL-female group. (CTRL-male group, n = 8; SPS-male group, n = 8; CTRL-female group, n = 11; SPS-female group, n = 12).

Exposure to SPS induced a long-term increase of avoidance, arousal, repetitive and anxiety-like behavior in male but not female rats

We examined whether SPS exposure altered avoidance and anxiety-like behavior in the elevated plus maze long-after trauma in males and females. As shown in Fig.3a, two-way ANOVA for time spent in the open arms indicated a strong tendency towards significance for SPS ($F_{(1,37)}$ = 3.582; P = 0.066) no significant effects of sex ($F_{(1,37)}$ = 0.922; P = 0.343), and a significant interaction between these two factors ($F_{(1,37)}$ = 4.774; P = 0.035). Post-hoc analysis revealed that SPS-male rats spent less time in the

open arms compared to their control group ($P < 0.05$). Two-way ANOVA for the number of entries in the open arms showed a tendency towards significance for SPS ($F_{(1,37)} = 2.403$; $P = 0.130$), no significant effects of sex ($F_{(1,37)} = 0.980$; $P = 0.329$), and a significant interaction between these two factors ($F_{(1,37)} = 4.455$; $P = 0.042$; Fig.3b). Post hoc analysis indicated that SPS-male rats presented less open arm entries compared to CTRL-male rats ($P < 0.05$). No post hoc comparisons were found between SPS-female and CTRL-female rats. These results indicate that SPS exposure induced a persistent increase in avoidance and anxiety-like behavior in the elevated plus maze in male but not in female rats.

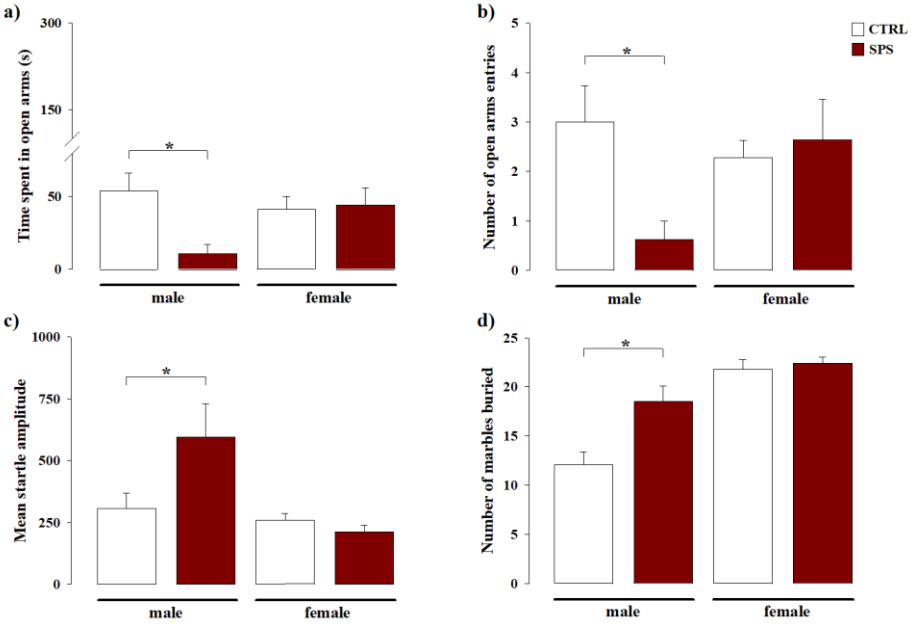
Acoustic startle response

To assess long-term effects induced by SPS on hyperarousal and anxious-like behavior in male and female rats, acoustic startle response was performed. As shown in Fig.3c, two-way ANOVA for mean startle amplitude showed a tendency towards significance for SPS ($F_{(1,38)} = 3.612$; $P = 0.065$), a significant effect of sex ($F_{(1,38)} = 11.551$; $P = 0.002$) and a significant SPS x sex interaction ($F_{(1,38)} = 6.931$; $P = 0.012$). Post hoc analysis revealed that SPS-male rats exhibited higher mean startle amplitude than the correspondent control group ($P < 0.05$). No post hoc comparisons were found between SPS-female and CTRL-female rats. Thus, these results indicating that SPS induced persistent hyperarousal and increased anxious-like behavior in males but not in females 30 days after trauma exposure.

Marble burying

We evaluated whether SPS exposure induced long-term avoidance, anxious- and compulsive-like behaviors in male and female rats in a marble burying test. As shown in Fig.3d, two-way ANOVA for the number of marbles buried indicated significant effects of SPS ($F_{(1,34)} = 7.113$; $P = 0.012$), sex ($F_{(1,34)} = 29.883$; $P < 0.0001$) and

interaction between these two factors ($F_{(1,34)}$ = 5.473; P = 0.025). Post hoc analysis revealed that the number of marbles buried by SPS-male rats was higher than in control group (P < 0.05). No post hoc comparisons were found between SPS-female and CTRL-female rats. These results indicate that SPS exposure selectively increased avoidance in male rats 30 days after trauma.



Long-term effects of SPS on anxiety-like behavior, hyperarousal, avoidance and repetitive behaviors in the elevated plus maze, acoustic startle response and marble burying tasks. Exposure to SPS selectively induced anxiety-like behavior, hyperarousal and increased avoidance in males but not in females, 30 days after trauma. Effects of SPS on (a) time spent in the open arms and (b) number of entries in the open arms in the elevated plus maze task; (c) mean startle amplitude in the acoustic startle response task; (d) number of marbles buried in the marble burying task. *, P < 0.05, vs. CTRL-male group. (CTRL-male group, n = 11; SPS-male group, n = 8; CTRL-female group, n = 11; SPS-female group, n = 11/12 in the elevated plus maze and acoustic startle response tasks, respectively, or CTRL-male group, n = 11; SPS-male group, n = 8; CTRL-female group, n = 9; SPS-female group, n = 10 in the marble burying task.

Exposure to SPS did not induce long-term spatial memory dysfunctions in male and female rats

This experiment examined whether SPS induced long-term alterations of spatial memory in male and female rats in a Morris water maze task. As shown in Fig.4a, RM ANOVA for escape latency during the 3 days of spatial training revealed significant effects of trials ($F_{(11,385)}$ = 30.811; P < 0.0001), indicating that all groups of rats progressively learned to locate the platform across the 3 training sessions (no other statistical differences were found). Two-way ANOVA for initial latency to cross the platform location indicated a tendency towards significance for SPS ($F_{(1,35)}$ = 1.897; P = 0.177), no significant effects of sex ($F_{(1,35)}$ = 0.107; P = 0.746), but a significant interaction between these two factors ($F_{(1,35)}$ = 7.710; P = 0.009; Fig.4b). Post hoc analysis revealed that the latency to cross the platform location of SPS-male rats was higher than that of the control group (P < 0.05). Two-way ANOVA for the number of crossings through the platform location revealed a significant effect of SPS ($F_{(1,35)}$ = 4.115; P = 0.050), but no significant effects of sex ($F_{(1,35)}$ = 0.020; P = 0.889) and a tendency towards significance for the SPS x sex interaction ($F_{(1,35)}$ = 2.598; P = 0.116; Fig.4c). Post hoc analysis indicated that the frequency of crossings through the platform location of SPS-male rats was lower than those of controls (P < 0.05). These results show that exposure to SPS induced long-term alterations of spatial memory retention in male but not female rats.

Exposure to SPS reduced fear extinction retrieval in female but not in male rats

To examine whether SPS induced long-term alterations of fear memory dynamics, an auditory fear conditioning task was performed in male and female rats 30 days after trauma exposure. RM ANOVA for the percentage of freezing during fear conditioning on day 1 (Fig.4d) revealed significant effects of tone presentation ($F_{(1,34)}$ = 6.757; P < 0.0001) and SPS x tone presentation interaction ($F_{(1,34)}$ = 6.757; P = 0.014), but no significant effects of SPS ($F_{(1,34)}$ = 0.866; P = 0.359), sex ($F_{(1,34)}$ = 0.100; P = 0.754),

SPS x sex interaction ($F_{(1,34)}$ = 0.604; P = 0.442), sex x tone presentation interaction ($F_{(1,34)}$ = 1.701; P = 0.201) or SPS x sex x tone presentation interaction ($F_{(1,34)}$ = 0.043; P = 0.838). Two-way ANOVA for time spent in freezing during the memory extinction phase on day 2 (Fig.4e) did not reveal any significant effects of SPS ($F_{(1,34)}$ = 0.323; P = 0.574), sex ($F_{(1,34)}$ = 0.450; P = 0.507) or SPS x sex interaction ($F_{(1,34)}$ = 0.013; P = 0.910). Two-way ANOVA for time spent in freezing during the memory retrieval session on day 3 (Fig.4f), did not show significant effects of SPS ($_{(1,34)}$ = 0.267; P = 0.608) or sex ($F_{(1,34)}$ = 0.031; P = 0.862), but a significant of SPS x sex interaction effect ($F_{(1,34)}$ = 4.579; P = 0.040). Post hoc analysis showed a lower percentage of freezing in female rats exposed to SPS as compared to control female rats (P < 0.05). These results indicate that SPS altered extinction retrieval 30 days after trauma only in female rats.

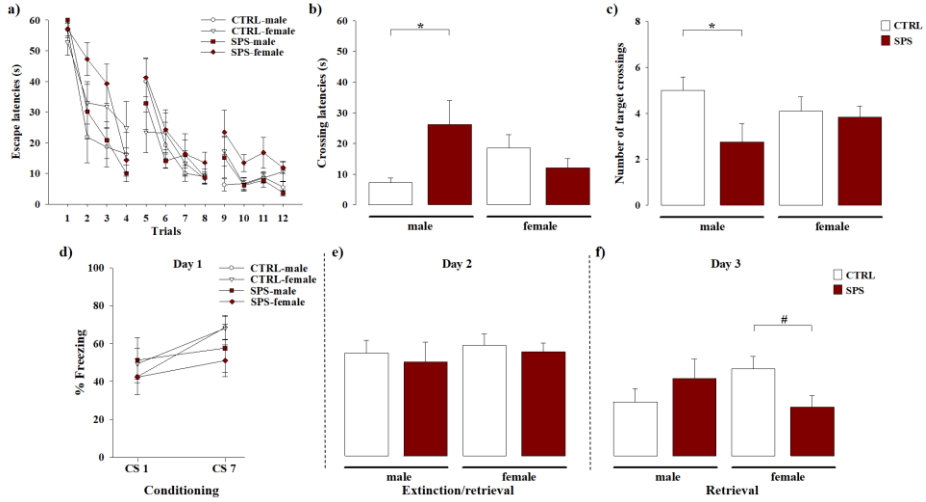


Figure 4: Long-term sex-divergent effects of SPS on spatial and fear memory in the Morris water maze and in the auditory fear conditioning tasks. Exposure to SPS impaired spatial memory retention in males but not females in the Morris water maze task while it facilitated fear extinction retrieval in the auditory fear conditioning task selectively in females but not in males. Effects of SPS in the Morris water maze on (a) escape latencies during the 4 trials of the

3 training days, (b) latency to cross the platform location and (c) number of crossings through the platform location during the probe session. Effects of SPS in the fear conditioning paradigm on the (d) percentage of time spent in freezing during the first and the last tone presentations at conditioning (day 1), (e) average of percentage of time spent in freezing during all 12 trials at extinction (day 2), (f) average of percentage of time spent in freezing during all 8 trials at extinction retrieval (day 3). *, $P < 0.05$, vs. CTRL-male (Morris water maze) or #, $P < 0.05$ vs. CTRL-female (fear conditioning) groups. (CTRL-male group, n = 8; SPS-male group, n = 8; CTRL-female group, n = 11; SPS-female group, n = 12 in the Morris water maze or CTRL-male group, n = 11; SPS-male group, n = 8; CTRL-female group, n = 9; SPS-female group, n = 10 in the auditory fear conditioning).

Discussion

The present findings indicate that SPS exposure induced long-term sex-divergent alterations in adult rats. In male rats, we observed emotional dysfunctions, while fear memory dynamics were unaffected. Contrarily, in females, exposure to SPS augmented extinction of cued fear memory, while leaving emotional behavior unaltered. Interestingly, both sexes presented reduced exploratory behavior long after stress exposure. It is important to note that emotional and spatial memory alterations (i.e. increased avoidance, anxiety-like behavior, hyperarousal, impaired memory retention) were observed a month after the SPS exposure in males, thus indicating that, at least in males, this model could recapitulate the emotional dysfunctions seen in PTSD, which develop and persist long after the traumatic experience in humans. Interestingly, in referring to the cognitive domain SPS impaired spatial memory retention but not cued fear memory processes examined in this study, in males. SPS has been extensively shown to induce behavioral and endocrine effects resembling the hallmark symptoms observed in PTSD patients [7,13,14,21] and, similarly to PTSD, the manifestation of these effects is time-dependent and requires a 1-2 week incubation period [22,25,26]. However, the majority of the studies using SPS to model the main traits of PTSD in rodents did not test whether behavioral alterations persisted long

after trauma (Iwamoto et al., 2007; Souza et al., 2017; Lisieski et al., 2018; Verbitsky et al., 2020). Thus, our study provides important evidence that SPS is able to induce long-term emotional alterations in males, modeling the chronicity of PTSD-induced anxiety symptoms, hyperarousal and avoidance behavior [1,50], which gives a great relevance and significant translational value to our results. Specifically, we found that 30 days after SPS exposure, male rats showed increased anxiety in the elevated plus maze test (e.g. less time spent and reduced entries in the open arms), exhibited higher mean startle response amplitude and buried more marbles in the marble burying task compared to controls, thus indicating an altered emotional and avoidance domain long-after trauma. This is consistent with a previous study showing in rats enhanced anxious-like behaviors in the elevated plus maze task 4 weeks after SPS exposure [29]. Furthermore, although only 1 week after SPS, other preclinical studies have also demonstrated enhanced acoustic startle response [16,20] and avoidance in the marble burying task in male rats [51]. In addition to emotional alterations, PTSD patients present impaired cognitive functions, including spatial memory alterations [1,2,52]. Previous studies found similar alterations in male rodents tested 1 or 2 weeks after SPS exposure [7,11,13,14]. Our findings add to the current knowledge indicating that in male spatial memory alterations last long after trauma. Interestingly, we found that 1 month after trauma exposure cued fear memory processes of SPS-exposed male rats did not differ from that of controls in the auditory fear conditioning task. Consistently, previous evidence has shown that SPS does not produce alterations in a fear conditioning paradigm 4 weeks after trauma [29]. Recent findings from our laboratory have indicated that locomotor activity is strongly related to maladaptive changes in a rat model for PTSD [41]. Here we found that both sexes presented reduced motor activity in an open field task one month after SPS. This reduced exploration of a novel environment (i.e. open field arena) is suggestive of stress hyper-responsiveness and fear generalization. Interestingly, a previous study in males showed that SPS-induced reduction in locomotor activity did not persist over 4 weeks, but accordingly with our findings the same study reported long-term increased anxiety-like behavior in an

elevated plus maze [29]. Quite surprisingly, besides the reduction of locomotor activity, we found that, overall, SPS did not induce a persistent PTSD-like phenotype in females. The few studies examining the effects of SPS in females measured behavioral outcomes 1-2 weeks after trauma exposure [35–39]. We provide the first evidence that, contrarily to what we found in males, SPS did not induce any long-term behavioral alterations in the elevated plus maze, acoustic startle response, marble burying and Morris water maze tasks, 1 month after trauma. On the other hand, our results showed that females exposed to SPS presented less freezing behavior at extinction retrieval compared to controls, thus indicating reduced fear retrieval and increased fear extinction. Supporting our findings, previous research has demonstrated that 1 week after SPS, male rats exhibited enhanced acoustic startle response, whereas females' response was unaffected [35,36]. The same effects were also observed in human PTSD patients [34]. Long-term effects of SPS on fear memory processes in female rats have not been extensively explored yet. To our knowledge, only two preclinical studies have evaluated the effects on fear memory after SPS in females and reported contrasting findings. One study showed reduced extinction recall in males but no changes in females [38] and another report found impaired extinction and enhanced fear retrieval in both sexes [39]. In both studies, however, memory alterations were evaluated only within 1 week post-SPS exposure. Thus, in the light of our current results, it appears that the SPS model in females is ineffective to mimicking persistent alterations of fear memory processes observed in PTSD patients, rather our results indicate that SPS exposure promotes fear memory extinction of a second stressful event experienced 1 month after SPS. In conclusion, our data provide the first evidence that the ability of the SPS model to resemble the chronicity of PTSD-like symptomatology is sex-dependent, with SPS-exposed males showing long-term reduced locomotion, hyperarousal, increased avoidance, anxiety-like behavior and spatial memory retention deficits and SPS-exposed females only presenting reduced locomotion and increased fear extinction 1 month after trauma. Clinical literature indicates that women are twice as likely to suffer from PTSD compared to men [4–6]

and it is still not clearly understood whether this higher prevalence in women is related to a higher susceptibility to PTSD development [53]. Our results provide evidence supporting fundamental sex-differences in the response to a traumatic event in rats and the subsequent susceptibility to develop a PTSD-like symptomatology. Thus, our findings are relevant to future research aimed not only at investigating sex-differences in the neurobiology of trauma-related disorders, but also at evaluating pharmacological interventions to treat long-term emotional alterations associated with PTSD.

Conclusions

Our findings demonstrate that SPS induces in rats long-term emotional and cognitive alterations resembling some of the core symptoms of PTSD and that such effects are sex divergent. While male rats exposed to SPS display reduced locomotion, hyperarousal, increased avoidance, anxiety-like behavior and spatial memory retention deficits, SPS-exposed females only show reduced locomotion and altered fear extinction recall 30 days after trauma. To our knowledge, this is the first evidence that the long-term effects induced by acute trauma in rodents are sex divergent. Clinical evidence demonstrate that men affected by PTSD tend to develop more "externalizing" symptoms while women affected by PTSD more "internalizing" ones. Our study thus opens the way to additional studies urgently needed to clarify the neurobiological underpinnings of gender divergent symptoms after trauma exposure.

References

[1] American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Association, 2013. https://doi.org/10.1176/appi.books.9780890425596.

[2] R. Yehuda, C.W. Hoge, A.C. McFarlane, E. Vermetten, R.A. Lanius, C.M. Nievergelt, S.E. Hobfoll, K.C. Koenen, T.C. Neylan, S.E. Hyman, Post-traumatic stress disorder, Nat. Rev. Dis. Prim. 1 (2015). https://doi.org/10.1038/nrdp.2015.57.

[3] H. Javidi, M. Yadollahie, Post-traumatic Stress Disorder, Int J Occup Environ Med. 3 (2012) 2–9.

[4] R.C. Kessler, A. Sonnega, E. Bromet, M. Hughes, C.B. Nelson, Posttraumatic Stress Disorder in the National Comorbidity Survey, Arch. Gen. Psychiatry. 52 (1995) 1048–1060. https://doi.org/10.1001/archpsyc.1995.03950240066012.

[5] N. Breslau, Gender differences in trauma and posttraumatic stress disorder, J. Gender-Specific Med. 5 (2002) 34–40.

[6] S. Seedat, D.J. Stein, Trauma and post-traumatic stress disorder in women: A review, Int. Clin. Psychopharmacol. 15 (2000).

[7] A. Verbitsky, D. Dopfel, N. Zhang, Rodent models of post-traumatic stress disorder: behavioral assessment, Transl. Psychiatry. 10 (2020). https://doi.org/10.1038/s41398-020-0806-x.

[8] A. Berardi, V. Trezza, M. Palmery, L. Trabace, V. Cuomo, P. Campolongo, An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD), Front. Behav. Neurosci. 8 (2014) 1–12. https://doi.org/10.3389/fnbeh.2014.00142.

[9] G. Richter-Levin, Acute and long-term behavioral correlates of underwater trauma-potential relevance to stress and post-stress syndromes, Psychiatry Res. 79 (1998) 73–83. https://doi.org/10.1016/S0165-1781(98)00030-4.

[10] P.R. Zoladz, D.M. Diamond, Predator-based psychosocial stress animal model of PTSD: Preclinical assessment of traumatic stress at cognitive, hormonal, pharmacological, cardiovascular and epigenetic levels of analysis, Exp. Neurol. 284 (2016) 211–219. https://doi.org/10.1016/j.expneurol.2016.06.003.

[11] S. Yamamoto, S. Morinobu, S. Takei, M. Fuchikami, A. Matsuki, S. Yamawaki, I. Liberzon, Single prolonged stress: Toward an animal model of posttraumatic stress disorder, Depress. Anxiety. 26 (2009) 1110–1117. https://doi.org/10.1002/da.20629.

[12] I. Liberzon, M. Krstov, E.A. Young, Stress-restress: Effects on ACTH and fast feedback, Psychoneuroendocrinology. 22 (1997) 443–453. https://doi.org/10.1016/S0306-4530(97)00044-9.

[13] M.J. Lisieski, A.L. Eagle, A.C. Conti, I. Liberzon, S.A. Perrine, Single-prolonged stress: A review of two decades of progress in a rodent model of post-traumatic stress disorder, Front. Psychiatry. 9 (2018). https://doi.org/10.3389/fpsyt.2018.00196.

[14] R.R. Souza, L.J. Noble, C.K. McIntyre, Using the single prolonged stress model to examine the pathophysiology of PTSD, Front. Pharmacol. 8 (2017). https://doi.org/10.3389/fphar.2017.00615.

[15] F. Han, J. Ding, Y. Shi, Expression of amygdala mineralocorticoid receptor and glucocorticoid receptor in the single-prolonged stress rats, BMC Neurosci. 15 (2014). https://doi.org/10.1186/1471-2202-15-77.

[16] S. Khan, I. Liberzon, Topiramate attenuates exaggerated acoustic startle in an animal model of PTSD, Psychopharmacology (Berl). 172 (2004) 225–229. https://doi.org/10.1007/s00213-003-1634-4.

[17] F.F. Liu, L.D. Yang, X.R Sun, H. Zhang, W. Pan, X.M. Wang, J.J. Yang, M.H. Ji, H.M. Yuan, NOX2 Mediated-Parvalbumin Interneuron Loss Might Contribute to Anxiety-Like and Enhanced Fear Learning Behavior in a Rat Model of Post-Traumatic Stress Disorder, Mol. Neurobiol. 53 (2016) 6680–6689. https://doi.org/10.1007/s12035-015-9571-x.

[18] Y.L. Miao, W.Z. Guo, W.Z. Shi, W.W. Fang, Y. Liu, J. Liu, B.W. Li, W. Wu, Y.F. Li, Midazolam ameliorates the behavior deficits of a rat posttraumatic stress disorder model through dual 18 kDa translocator protein and central benzodiazepine receptor and neurosteroidogenesis, PLoS One. 9 (2014). https://doi.org/10.1371/journal.pone.0101450.

[19] S.M. Keller, W.B. Schreiber, B.R. Stanfield, D. Knox, Inhibiting corticosterone synthesis during fear memory formation exacerbates cued fear extinction memory deficits within the single prolonged stress model, Behav. Brain Res. 287 (2015) 182–186. https://doi.org/10.1016/j.bbr.2015.03.043.

[20] K. Kohda, K. Harada, K. Kato, A. Hoshino, J. Motohashi, T. Yamaji, S. Morinobu, N. Matsuoka, N. Kato, Glucocorticoid receptor activation is involved in producing abnormal phenotypes of single-prolonged stress rats: A putative post-traumatic stress disorder model, Neuroscience. 148 (2007) 22–33. https://doi.org/10.1016/j.neuroscience.2007.05.041.

[21] Y. Iwamoto, S. Morinobu, T. Takahashi, S. Yamawaki, Single prolonged stress increases contextual freezing and the expression of glycine transporter 1 and vesicle-associated membrane protein 2 mRNA in the hippocampus of rats, Prog. Neuro-Psychopharmacology Biol. Psychiatry. 31 (2007) 642–651. https://doi.org/10.1016/j.pnpbp.2006.12.010.

[22] D. Knox, S.A. George, C.J. Fitzpatrick, C.A. Rabinak, S. Maren, I. Liberzon, Single prolonged stress disrupts retention of extinguished fear in rats, Learn. Mem. 19 (2012) 43–49. https://doi.org/10.1101/lm.024356.111.

[23] Y. Wen, B. Li, F. Han, E. Wang, Y. Shi, Dysfunction of calcium/calmodulin/CaM kinase IIα cascades in the medial prefrontal cortex in post-traumatic stress disorder, Mol. Med. Rep. 6 (2012) 1140–1144. https://doi.org/10.3892/mmr.2012.1022.

[24] W.M. Vanderheyden, S.A. George, L. Urpa, M. Kehoe, I. Liberzon, G.R. Poe, Sleep alterations following exposure to stress predict fear-associated memory impairments in a rodent model of PTSD, Exp. Brain Res. 233 (2015) 2335–2346. https://doi.org/10.1007/s00221-015-4302-0.

[25] I. Liberzon, J.F. López, S.B. Flagel, D.M. Vázquez, E.A. Young, Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: Relevance to post-traumatic stress disorder, J. Neuroendocrinol. 11 (1999) 11–17. https://doi.org/10.1046/j.1365-2826.1999.00288.x.

[26] Z. Wu, Q. Tian, F. Li, J. Gao, Y. Liu, M. Mao, J. Liu, S. Wang, G. Li, D. Ge, Y. Mao,

W. Zhang, Z. Liu, Y. Song, Behavioral changes over time in post-traumatic stress disorder: Insights from a rat model of single prolonged stress, Behav. Processes. 124 (2016) 123–129. https://doi.org/10.1016/j.beproc.2016.01.001.

[27] S.E. Sillivan, N.F. Joseph, S. Jamieson, M.L. King, I. Chévere-Torres, I. Fuentes, G.P. Shumyatsky, A.F. Brantley, G. Rumbaugh, C.A. Miller, Susceptibility and Resilience to Posttraumatic Stress Disorder–like Behaviors in Inbred Mice, Biol. Psychiatry. 82 (2017) 924–933. https://doi.org/10.1016/j.biopsych.2017.06.030.

[28] D. Toledano, P. Gisquet-Verrier, Only susceptible rats exposed to a model of PTSD exhibit reactivity to trauma-related cues and other symptoms: An effect abolished by a single amphetamine injection, Behav. Brain Res. 272 (2014) 165–174. https://doi.org/10.1016/j.bbr.2014.06.039.

[29] D. Feng, B. Guo, G. Liu, B. Wang, W. Wang, G. Gao, H. Qin, S. Wu, FGF2 alleviates PTSD symptoms in rats by restoring GLAST function in astrocytes via the JAK/STAT pathway, Eur. Neuropsychopharmacol. 25 (2015) 1287–1299. https://doi.org/10.1016/j.euroneuro.2015.04.020.

[30] R.C. Kessler, S. Aguilar-Gaxiola, J. Alonso, C. Benjet, E.J. Bromet, G. Cardoso, L. Degenhardt, G. de Girolamo, R. V. Dinolova, F. Ferry, S. Florescu, O. Gureje, J.M. Haro, Y. Huang, E.G. Karam, N. Kawakami, S. Lee, J.P. Lepine, D. Levinson, F. Navarro-Mateu, B.E. Pennell, M. Piazza, J. Posada-Villa, K.M. Scott, D.J. Stein, M. Ten Have, Y. Torres, M.C. Viana, M. V. Petukhova, N.A. Sampson, A.M. Zaslavsky, K.C. Koenen, Trauma and PTSD in the WHO World Mental Health Surveys, Eur. J. Psychotraumatol. 8 (2017). https://doi.org/10.1080/20008198.2017.1353383.

[31] M.W. King, A.E. Street, J.L. Gradus, D.S. Vogt, P.A. Resick, Gender Differences in Posttraumatic Stress Symptoms Among OEF/OIF Veterans: An Item Response Theory Analysis, J. Trauma. Stress. 26 (2013) 175–183. https://doi.org/10.1002/jts.21802.

[32] H. Cohen, N. Kozlovsky, C. Alona, M.A. Matar, Z. Joseph, Animal model for PTSD: From clinical concept to translational research, Neuropharmacology. 62 (2012) 715–724. https://doi.org/10.1016/j.neuropharm.2011.04.023.

[33] R.M. Shansky, Sex differences in PTSD resilience and susceptibility: Challenges for animal models of fear learning, Neurobiol. Stress. 1 (2015) 60–65. https://doi.org/10.1016/j.ynstr.2014.09.005.

[34] A.M. Medina, V.Y. Mejia, A.M. Schell, M.E. Dawson, G. Margolin, Startle reactivity and PTSD symptoms in a community sample of women., Psychiatry Res. 101 (2001) 157–169.

[35] A.E. Pooley, R.C. Benjamin, S. Sreedhar, A.L. Eagle, A.J. Robison, M.S. Mazei-Robison, S.M. Breedlove, C.L. Jordan, Sex differences in the traumatic stress response: PTSD symptoms in women recapitulated in female rats, Biol. Sex Differ. 9 (2018). https://doi.org/10.1186/s13293-018-0191-9.

[36] A.E. Pooley, R.C. Benjamin, S. Sreedhar, A.L. Eagle, A.J. Robison, M.S. Mazei-

Robison, S.M. Breedlove, C.L. Jordan, Sex differences in the traumatic stress response: The role of adult gonadal hormones, Biol. Sex Differ. 9 (2018). https://doi.org/10.1186/s13293-018-0192-8.

[37] R.J. Nahvi, C. Nwokafor, L.I. Serova, E.L. Sabban, Single prolonged stress as a prospective model for posttraumatic stress disorder in females, Front. Behav. Neurosci. 13 (2019). https://doi.org/10.3389/fnbeh.2019.00017.

[38] S.M. Keller, W.B. Schreiber, J.M. Staib, D. Knox, Sex differences in the single prolonged stress model, Behav. Brain Res. 286 (2015) 29–32. https://doi.org/10.1016/j.bbr.2015.02.034.

[39] T.M. Zer-Aviv, I. Akirav, Sex differences in hippocampal response to endocannabinoids after exposure to severe stress, Hippocampus. 26 (2016) 947–957. https://doi.org/10.1002/hipo.22577.

[40] E. Ganon-Elazar, I. Akirav, Cannabinoids prevent the development of behavioral and endocrine alterations in a rat model of intense stress, Neuropsychopharmacology. 37 (2012) 456–466. https://doi.org/10.1038/npp.2011.204.

[41] P. Colucci, E. Marchetta, G.F. Mancini, P. Alva, F. Chiarotti, M.T. Hasan, P. Campolongo, Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD), Transl. Psychiatry. 10 (2020). https://doi.org/10.1038/s41398-020-00929-9.

[42] N. Almenrader, P. Colucci, V. De Castro, D. Valeri, M. Palmery, V. Trezza, P. Campolongo, Effects of sevoflurane and clonidine on acid base status and long-term emotional and cognitive outcomes in spontaneously breathing rat pups, PLoS One. 12 (2017). https://doi.org/10.1371/journal.pone.0173969.

[43] M. Morena, K.D. Leitl, H.A. Vecchiarelli, J.M. Gray, P. Campolongo, M.N. Hill, Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety., Neuropharmacology. 111 (2016) 59–69. https://doi.org/10.1016/j.neuropharm.2016.08.020.

[44] M.T. Zanda, P. Fadda, S. Antinori, M. Di Chio, W. Fratta, C. Chiamulera, L. Fattore, Methoxetamine affects brain processing involved in emotional response in rats, Br. J. Pharmacol. 174 (2017) 3333–3345. https://doi.org/10.1111/bph.13952.

[45] C. Scuderi, C. Stecca, M. Valenza, P. Ratano, M.R. Bronzuoli, S. Bartoli, L. Steardo, E. Pompili, L. Fumagalli, P. Campolongo, L. Steardo, Palmitoylethanolamide controls reactive gliosis and exerts neuroprotective functions in a rat model of Alzheimer's disease, Cell Death Dis. 5 (2014). https://doi.org/10.1038/cddis.2014.376.

[46] M. Morena, V. De Castro, J.M. Gray, M. Palmery, V. Trezza, B. Roozendaal, M.N. Hill, P. Campolongo, Training-associated emotional arousal shapes endocannabinoid modulation of spatial memory retrieval in rats, J. Neurosci. 35 (2015) 13962–13974. https://doi.org/10.1523/JNEUROSCI.1983-15.2015.

[47] J. Rodriguez-Romaguera, F. Sotres-Bayon, D. Mueller, G.J. Quirk, Systemic Propranolol Acts Centrally to Reduce Conditioned Fear in Rats Without Impairing Extinction, Biol. Psychiatry. 65 (2009) 887–892. https://doi.org/10.1016/j.biopsych.2009.01.009.

[48] P. Atsak, D. Hauer, P. Campolongo, G. Schelling, R. V. Fornari, B. Roozendaal, Endocannabinoid Signaling within the Basolateral Amygdala Integrates Multiple Stress Hormone Effects on Memory Consolidation, Neuropsychopharmacology. 40 (2015) 1485–1494. https://doi.org/10.1038/npp.2014.334.

[49] D.C. Blanchard, R.J. Blanchard, Innate and conditioned reactions to threat in rats with amygdaloid lesions, J. Comp. Physiol. Psychol. 81 (1972) 281–290. https://doi.org/10.1037/h0033521.

[50] R.K. Pitman, A.M. Rasmusson, K.C. Koenen, L.M. Shin, S.P. Orr, M.W. Gilbertson, M.R. Mila, I. Liberzon, Biological studies of post-traumatic stress disorder, Nat. Rev. Neurosci. 13 (2012) 769–787. https://doi.org/10.1038/nrn3339.

[51] L.J. Noble, I.J. Gonzalez, V.B. Meruva, K.A. Callahan, B.D. Belfort, K.R. Ramanathan, E. Meyers, M.P. Kilgard, R.L. Rennaker, C.K. McIntyre, Effects of vagus nerve stimulation on extinction of conditioned fear and post-traumatic stress disorder symptoms in rats, Transl. Psychiatry. 7 (2017) e1217. https://doi.org/10.1038/tp.2017.191.

[52] J.K. Miller, S. McDougall, S. Thomas, J.M. Wiener, Impairment in active navigation from trauma and Post-Traumatic Stress Disorder, Neurobiol. Learn. Mem. 140 (2017) 114–123. https://doi.org/10.1016/j.nlm.2017.02.019.

[53] D.F. Tolin, E.B. Foa, Sex differences in trauma and posttraumatic stress disorder: A quantitative review of 25 years of research, Psychol. Bull. 132 (2006) 959–992. https://doi.org/10.1037/0033-2909.132.6.959.

CHAPTER 4

Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder

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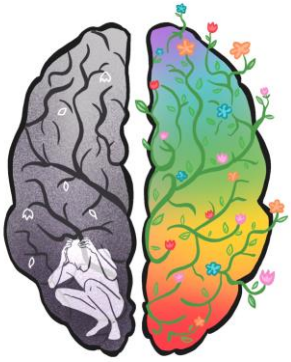

Abstract

Post-traumatic stress disorder (PTSD) is a psychiatric disorder whose pathogenesis relies on a maladaptive expression of the memory for a life-threatening experience, characterized by over-consolidation, generalization, and impaired extinction, which are responsible of dramatic changes in arousal, mood, anxiety, and social behavior. Even if subjects experiencing a traumatic event during lifetime all show an acute response to the trauma, only a subset of them (susceptible) ultimately develops PTSD, meanwhile the others (resilient) fully recover after the first acute response. However, the dynamic relationships between the interacting brain circuits that might potentially link trauma-related experiences to the emergence of susceptible and resilient PTSD phenotypes in individuals is not well understood. Toward the first step to reach this goal, we have implemented our experimental PTSD model previously developed, making it suitable to differentiate between susceptible (high responders, HR) and resilient (low responders, LR) rats in terms of over-consolidation, impaired extinction, and social impairment long after trauma. Rats were exposed to five footshocks paired with social isolation. One week after trauma but before extinction, animals were tested in the Open Field and Social Interaction tasks for the identification of a predictive variable to identify susceptible and resilient animals before the possible appearance of a PTSD-like phenotype. Our findings show that exploratory activity after trauma in a novel environment is a very robust variable to predict susceptibility towards a PTSD-like phenotype. This experimental model is thus able to screen and differentiate, before extinction learning and potential therapeutic intervention, susceptible and resilient PTSD-like rats.

Introduction

Post-traumatic stress disorder (PTSD) is a pathology with high prevalence and morbidity, the abnormal adaptation to a traumatic experience represents its specific pathogenic starting point[1–3]. The formation, consolidation, retrieval, and extinction of fear memories associated to a traumatic situation are orchestrated by multiple brain regions that act in a coordinate manner to elaborate the acute response to trauma[4–6]. After this normal acute response, most subjects fully recover. However, after experiencing a life-threatening event, in certain conditions the brain may switch to a maladaptive expression of memory specificities characterized by over-consolidation, memory generalization, and impaired extinction[7,8]. These cognitive alterations lead to a persistent reminding of the traumatic memories which in turn is responsible of PTSD development and consecutive symptoms (e.g., dramatic changes in arousal, mood, and social behavior)[1,3]. It has been estimated that more than 50% of the world population will encounter a trauma-causing experience once in their lifetime[9] and even if all the traumatize persons will show an acute response to the trauma, the majority of them will recover without intervention[10]. Therefore, an important question arises on why some people do develop PTSD after a life-threatening experience whereas others do not. It is well known that stress response cannot be sustained for a long time, the organism thus needs to develop effective physiological and psychological changes to cope with it[11]. Stressful experiences lead indeed to adaptation, but in some susceptible individuals this can be pathogenic[12,13]. The understanding of the neuronal mechanisms sustaining such pathogenic adaptation in susceptible subjects would hold the key for circuit-targeted therapeutics, especially due to the high rate of unresponsiveness or recrudescence following the initial remission of the pathology with the current treatments[14]. Animal models of psychiatric diseases are useful tools for understanding the pathophysiological mechanisms of a specific disorder, thus contributing in the development of more effective therapeutic strategies for humans[15]. Although there are different rodent PTSD models, they however lack good translational value[16,17]. These animal models reproduce only the emotional symptoms of PTSD, as hyperarousal and abnormal fear responses,

thus neglecting the etiological causes of the pathology such as the cognitive alterations. However, a valid PTSD animal model should be able to simultaneously capture both the cognitive and emotional features of the pathology. Another important aspect is the chronicity issue of the pathology to sustain the behavioral alterations in an animal model long after trauma which has high relevance and translational value[18]. However, long-term changes are rarely investigated possibly because the trauma experience is not long-lasting. Finally, the interindividual variability in response to trauma is of outmost importance. Unfortunately, almost all PTSD animal models homogenize all the trauma-exposed animals as having the same phenotype, regardless of susceptibility or resilience to develop a PTSD-like phenotype, thus lacking construct and predictive validity[19]. In the spare studies that consider the individual variability to trauma, only the anxiety symptoms are used to discern between different PTSD-like phenotypes[20,21], without considering the etiology (e.g. cognitive alterations such as the excessive memory consolidation and retrieval as well as the impaired extinction). Therefore, the development of an animal model which is able to predict individual differences in developing a chronic PTSD-like phenotype with regard of both cognitive and emotional alterations would be crucial to understand the neurobiological substrates underlying the individual variability towards PTSD development. We developed an animal model that is able to capture both cognitive etiological alterations of PTSD together with a peculiar set of emotional dysfunctions related to the social domain, alterations resembling some of the core symptoms observed in the human pathology[22]. Here, we applied our animal model to identify susceptible or resilient individuals towards PTSD development; our model identifies a robust predictive variable to rigorously and systematically screen and differentiate, before extinction learning and potential therapeutic intervention, between susceptible (high responders, HR) and resilient (low responders, LR) rats in terms of over-consolidation, impaired extinction, and social impairment.

Materials and methods

Animal care and use

A total of 124 male adult Sprague-Dawley rats (350–450 g at the time of testing), Charles River Laboratories (Calco, Italy), were kept in an air-conditioned colony room (temperature: 21 ± 1 °C; lights on from 07:00 AM to 7:00 PM) with pellet food and water available ad libitum. Each rat was randomly assigned to the behavioral procedures. For every experiment, the exact sample size for each experimental group/condition is indicated in the figure legends. Sample size was carried out considering a power = 80%, the probability of committing a first type error (alpha) equal to 0.05 (G-power statistics). The experiments were performed in Sprague-Dawley rats because in the present study we adapted the PTSD animal model we first described, in which is used the strain of rat[22,23]. All the experiments were performed during the light phase of the cycle. Rats were handled for 1 min for three consecutive days before behavioral testing. All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, and the D. L. 26/2014 of Italian Ministry of Health.

Behavioral procedures—Experiment 1

The PTSD model we first described[22,23] was adapted to make it suitable to distinguish between HR and LR animals in terms of over-consolidation, impaired extinction, and social impairment (for the experimental design see Fig. 1a). The experimental apparatus consisted in a metal trough shaped alley (60 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) connected to an animal shocker. All the experimental sessions were video-recorded and subsequently scored by two welltrained researchers blind to the experimental conditions. After each session, fecal boli were removed and the apparatus was cleaned with a 70% ethanol solution.

Housing

All rats were individually housed for 2 days prior to the habituation session and remained singly housed until the end of the behavioral testing. We have previously shown that social isolation is necessarily required to develop enduring signs of emotional distress upon exposure to a traumatic event[22].

Habituation

On the first day of testing (day −1), rats were individually taken from the home-cage and habituated for 5 min to the test apparatus. Rats were then returned to their home-cages.

Exposure session

On day 0, rats were individually placed in the apparatus for a total duration of 6 min, and were left undisturbed for the first 2 min. Then, 5 footshocks (2 s, 0.8 mA) were randomly delivered. After the last footshock rats were kept in the apparatus for 60 s to facilitate context association to the aversive stimuli. Screening of HR and LR populations. Five and 6 days after trauma exposure rats were subjected to the Open Field and Social Interaction tests, respectively, with the purpose of identify a predictive variable for the screening of HR and LR phenotypes in accordance to their behavior. Open Field test. Each rat was placed into the center of the Open Field arena made of black Plexiglas (80 × 80 × 60 cm) for 15 min. The test was performed under dim light conditions (2 lux). The total distance traveled (cm) by each rat, as well as the time spent in the center of the arena, were determined as parameters indicating the rat locomotor activity and emotionality, respectively. All the parameters here observed were acquired and analyzed using an automated video-tracking system (Smart, Panlab, Harvard Apparatus). After each session, rats were returned to their home-cage, fecal boli were removed, and the arena's walls and floor were cleaned with a 70% ethanol solution.

Social Interaction test

In the social Interaction test couples of rats were put for 10 min in a quadrangular arena (40 × 40 × 60 cm) made of plexiglass with clean sawdust covering the floor, under red light conditions. After each session, rats were returned to their home-cage, fecal boli were removed, sawdust was blended, and the arena's walls were cleaned with a 70% ethanol solution. Each couple of rats was established according to the unfamiliarity criteria (the two rats of each pair have never been housed in the same cage). In this test, social behavior was scored as previously described[22]. Extinction sessions and extinction retention test. Each of the four extinction sessions consisted in a 10-min re-exposure to the experimental apparatus, with the first carried out 7 days after the exposure session (day 7). Each subsequent session was separated from the preceding one by a 72-h interval (days 10, 13, and 16). During each extinction session the contextual freezing behavior was evaluated[24]. Particularly, the freezing behavior analyzed during the first extinction session (7 days after trauma exposure) was considered as an index of the overconsolidation of memory related to the traumatic experience, whereas the last extinction session (16 days after trauma exposure) was considered as the extinction retention test, and the contextual freezing behavior here evaluated was considered as an index of the impaired extinction in our PTSD animal model.

Social Interaction test

To evaluate the social impairment, rats were tested in the Social Interaction test, which was carried out 72 h after the extinction retention test (day 19). Couples of rats for this second Social Interaction test were different from the previous couples in the Social Interaction test at 6 days after trauma exposure. The test was conducted as described above.

Behavioral procedures—Experiment 2

In Experiment 2, a second group of rats was subjected to the rotarod test 5 days after trauma exposure (day 5), instead to the Open Field test, which was run 2 days before

the trauma (day −2), accordingly with the experimental design shown in Fig. 1b. The Open Field test and the other behavioral procedures (habituation, exposure, extinction sessions, extinction retention test and Social Interaction test) as well as the Housing condition were run and maintained at the same way as described for Experiment 1.

Rotarod test

The Rotarod test was performed as previously described[25]. The rotarod speed was regularly accelerated each 30 s from 10 to 60 rpm. The cut-off time was fixed at 300 s. Rats were given three trials with 30 min inter-trial rest intervals. The mean time taken from each animal to fall from the rotarod apparatus, across the three trials, was measured and it represented a measure of the motor activity, since it is the time taken by each rat to maintain its balance walking on the revolving rod.

Statistical analysis

Statistical analysis was performed using SPSS statistical software. Behavior was scored by three trained operators blinded to the animal phenotype and condition. Each measure is expressed as mean ± SEM. For each Pearson's correlation performed, R coefficient and the relative P value were evaluated and a good correlation between variables was considered for $R \geq 0.35$ and $P < 0.05$. Behavioral data were analyzed through a repeated measures ANOVA (RM ANOVA) or one-way ANOVA when appropriate. Tukey–Kramer post hoc test was performed to control for significant differences between groups and significance was considered for $P < 0.05$.

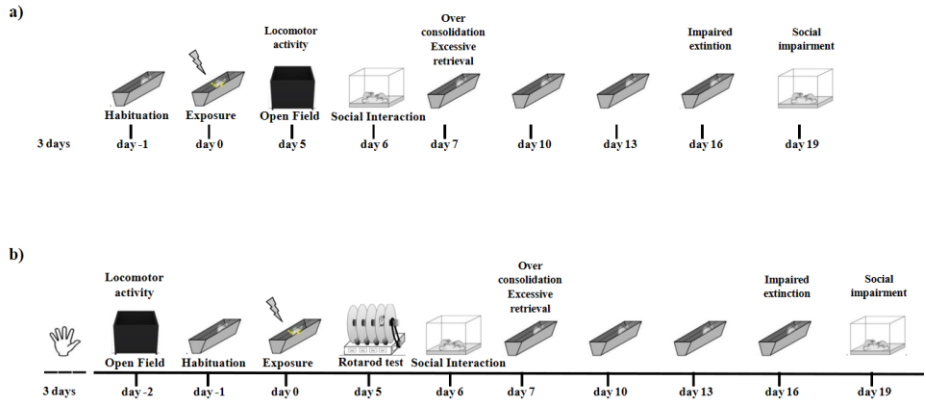

Figure 1: Experimental design. Behavioral procedures of Experiment 1 (a) and Experiment 2 (b).

Results

Exploratory activity after trauma is a reliable predictive variable to identify resilient and susceptible animals towards a PTSD-like phenotype. In the first experiment, we aimed at identifying a predictive variable for HR and LR phenotypes screening. To this aim we performed a correlation analysis between potentially predictive variables (total distance traveled in the Open Field test, time spent in the center of the arena in the Open Field test, and time spent in social interaction in the Social Interaction test performed before the extinction learning) and other behavioral outcomes associated with PTSD (such as over-consolidation, excessive retrieval, impaired extinction, and social alterations). Particularly, we found a significant negative correlation between the total distance traveled, which is an index of the exploratory activity, in the Open Field test performed 5 days after trauma exposure and the freezing behavior shown by rats during the first extinction session (day 7) ($R = -0.491$, $P < 0.0001$) (Fig. 2a), index of the over-consolidation of the trauma experience in the PTSD model, and the freezing behavior shown by rats during the extinction retention test (day 16) ($R = -0.532$, $P < 0.0001$) (Fig. 2b), index of the impaired extinction of the trauma experience in the PTSD model.

Thus, indicating that the less the rats explored the Open Field arena, the more they showed the freezing behavior during the first extinction session and the extinction retention test. Conversely, a significant positive correlation was found between the total distance traveled and the time spent in social in the Social Interaction test performed 19 days after trauma exposure ($R = 0.384$, $P < 0.001$) (Fig. 2c) as an index of social alterations in the PTSD model. Thus, indicating that the less the rats explored the Open Field arena, the less they spent time in interacting with a conspecific during the Social interaction test. Subsequently, we performed the same correlation analysis, taking in consideration, as a potentially predictive variable, the time spent in the center of the Open Field arena during the Open Field test performed 5 days after trauma exposure, and no significant correlations were found between this parameter and the freezing behavior shown by rats during the first extinction session (day 7) ($R = 0.011$, $P = 0.921$, Supplementary Fig. 1a), the freezing behavior shown by rats during the extinction retention test (day 16) ($R = 0.040$, $P = 0.727$, Supplementary Fig. 1b) and the time spent in social interaction in the Social Interaction test performed 19 days after trauma exposure ($R = 0.027$, $P = 0.812$, Supplementary Fig. 1c). Finally, we performed the correlation analysis taking in consideration the time spent by each rat in social interaction, during the Social Interaction test performed 6 days after trauma exposure, as a potentially predictive variable. The statistical analysis revealed no significant correlations between this factor and the freezing behavior shown by rats during the first extinction session (day 7) ($R = 0.107$, $P = 0.345$, Supplementary Fig. 1d), the freezing behavior shown by rats during the extinction retention test (day 16) ($R = 0.057$, $P = 0.617$, Supplementary Fig. 1e), and the time spent in social interaction in the Social Interaction test performed 19 days after trauma exposure ($R = 0.032$, $P = 0.776$, Supplementary Fig. 1f). For the statistical analysis of all the other parameters evaluated in the Open Field task, see Supplementary Results, Supplementary Figs. 2, 3, 4 and Supplementary Table 1.

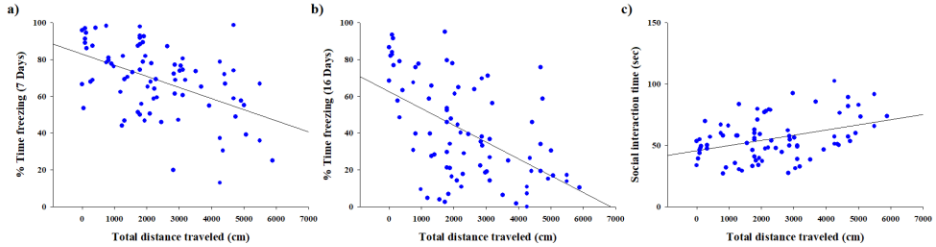

Figure 2: Exploratory activity after trauma is a robust predictive variable to identify resilient and susceptible animals towards a PTSD-like phenotype. The total distance traveled evaluated in the Open Field test performed 5 days after trauma exposure significantly correlated with behaviors at 7 and 16 days after trauma, indexes of the over-consolidation, and impaired extinction of memory for the traumatic experience, respectively (a, b) and with the social interaction time, evaluated in the Social Interaction test performed 19 days after trauma, index of social/emotional alterations in the PTSD-like phenotype (c). N = 80.

Rats classification according to their exploratory activity after trauma revealed behavioral alterations associated with the PTSD-like phenotype

Thereafter the identification of the total distance traveled in the Open Field test as a promising predictive variable for the HR and LR phenotypes screening, we defined the rats' classification according to the extremes (25th or 75th percentile) of the experimental group's distribution for this parameter. Each rat scored above 25th or over 75th percentiles has been considered as susceptible (HR) or resilient (LR), respectively, whereas each rat scored between 25th and 75th percentiles has been considered normal responder (NR). The subsequent RM ANOVA for the freezing behavior analysis across the extinction sessions in the three experimental groups (Fig. 3a) revealed a significant effect of the phenotype HR, LR, or NR ($F_{(2,77)} = 18.189$, $P < 0.0001$), a significant effect of the extinction sessions ($F_{(3,77)} = 48.510$, $P < 0.0001$) and tendency toward significance for the interaction between these two factors ($F_{(6,231)} = 2.071$, $P = 0.058$). Post hoc tests showed that HR rats presented higher levels of freezing across the extinction sessions and at extinction retention test as compared with the LR group (Ps < 0.01, for all days

of extinction), and they presented higher levels of freezing with respect to the NR group only during the third extinction session (day 13) and the extinction retention test (day 16) (Ps < 0.01). Moreover, the post hoc tests revealed that the LR group presented lower levels of freezing across all the extinction sessions as compared with the NR group (P < 0.05, days 7 and 13; P < 0.01, day 10), excepted for the extinction retention test (day 16) in which no significant differences between these two groups were found. Then, through a one-way ANOVA analysis, we analyzed the time spent in social interaction during the Social Interaction test performed 19 days after the trauma exposure (Fig. 3b) and a significant difference between the three experimental groups has been revealed ($F_{(2,77)}$ = 5.801, P = 0.005). Particularly, post hoc analysis revealed that HR and NR groups spent less time in social interaction with respect to the LR group (P < 0.01 and P < 0.05, respectively). The representative path tracks indicating the exploratory activity in the Open Field arena of HR, NR, and LR rats are shown in Fig. 3c. Trauma induced changes in the natural tendency to explore a new environment is a specific variable for the screening of a PTSD-like phenotype. In the second experiment, we aimed at evaluating whether is the pure motor activity to predict a PTSD-like phenotype rather than the trauma induced changes to exploratory activity. To this aim, we performed a correlation analysis between the mean time taken by each animal to fall from the rotarod apparatus (a pure measure of motor activity) and the behavioral outcomes associated with PTSD. The same correlation analysis was also performed between the total distance traveled in the Open Field test before trauma and the behavioral outcomes associated with the PTSD-like phenotype. The correlation analysis between the mean time across the three trials, for each animal to fall from the rotarod apparatus and the freezing behavior shown by rats during the first extinction session (day 7, Supplementary Fig. 5a) and the extinction retention test (day 16, Supplementary Fig. 5b) did not reveal any significant correlation (R = 0.133, P = 0.389; R = 0.106, P = 0.494; respectively). No significant correlation was also found between this parameter of pure motor activity and the time spent in social interaction during the Social Interaction test performed 19 days after trauma exposure (R = 0.073, P = 0.637,

Supplementary Fig. 5c). The correlation analysis between the total distance traveled in the Open Field arena before trauma and the behavioral outcomes associated with the PTSD-like phenotype did not revealed any significant correlation between this parameter and the freezing behavior shown during the first extinction session (day 7) (R = 0.047, P = 0.760, Supplementary Fig. 5d), the freezing behavior shown during the extinction retention test (day 16) (R = 0.036, P = 0.817, Supplementary Fig. 5e) and the time spent in social interaction during the Social Interaction test performed 19 days after trauma (R = 0.081, P = 0.600, Supplementary Fig. 5f). For the statistical analysis of all the other parameters evaluated in the Open Field task see Supplementary Results and Supplementary Table 2.

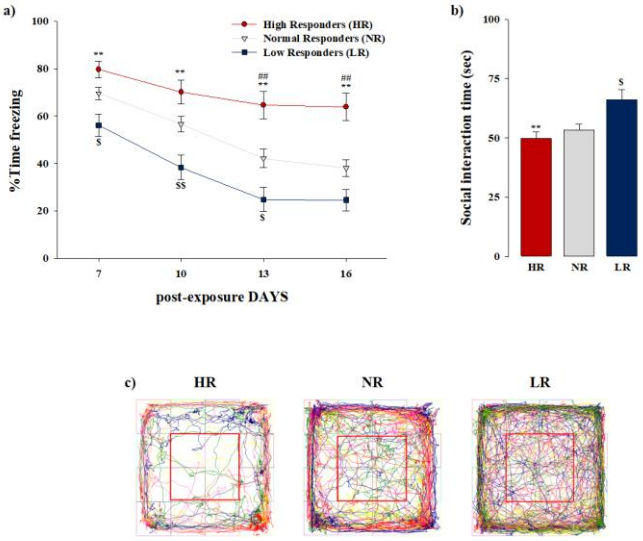

Figure 3: Rats classification according to their exploratory activity after trauma revealed behavioral alterations associated with the PTSD-like phenotype in rats. Freezing rates across the three extinction sessions (days: 7, 10, and 13) and the extinction retention test (day 16) in rats segregated in HR (susceptible), NR (normal), and LR (resilient) according to the 75th and 25th percentile of the experimental group's distribution for the total distance traveled in the Open Field test performed 5 days after trauma exposure. HR rats displayed increased freezing response compared to LR rats, across all the extinction sessions and still at extinction retention test, where

they showed higher level of freezing also with respect to the NR group. LR rats showed an exactly opposite freezing profile, with lower levels of freezing across all the extinction session and no significant differences with respect to the NR group at the extinction retention test. a Social interaction time of HR, NR, and LR rats. HR and NR rats spent less time interacting with a conspecific with respect to LR rats. b Representative path tracks of five animals per group indicating the exploratory activity in the Open Field arena of HR, NR, and LR rats (c). $^{\S}P < 0.05$ LR vs NR group; $^{**}P < 0.01$ HR vs LR group; $^{\#\#}P < 0.01$ HR vs NR group; $^{\S\S}P < 0.01$ LR vs NR group. N = 80 (20 HR, 40 NR, 20 LR).

Discussion

In the present study, by using specific behavioral tests suitable to map the individual differences in an experimental model of PTSD, we found a robust predictive variable for the screening of PTSD susceptibility immediately after trauma and before therapeutic intervention. We found the level of exploratory activity soon after trauma in a novel environment to be a robust and reliable predictive variable to identify later susceptibility towards PTSD development. The analysis of the locomotor activity in a novel arena is an easy and valuable parameter to simultaneously evaluate both motor activity and the natural tendency of rodents to explore a new environment[26]. Through a correlation analysis, we found a negative dependence between this parameter and all the memory alterations clusters in animals presenting a PTSD-like phenotype: i.e. over-consolidation and impaired extinction (represented by the percentage of time spent in freezing behavior, during the first extinction session 1 week after trauma, and after the last extinction session 16 days after trauma). Maladaptive memory processes are specific traits of PTSD pathology[2] which can be easily reproduced in rodents to investigate their neuronal underpinnings[22]. Contextual freezing behavior represents a fear and anxiety related response shown by rodents when exposed to the context in which they experienced trauma[27]. This behavior is linked to fear memory retention. The fear memory elicited during the first extinction session is a measure of memory retention and its retrieval. In fact, at this time point, the extinction learning has not

started yet, and an excessive memory retrieval can be due to a maladaptive memory consolidation processes induced by the traumatic experience and known as overconsolidation[28]. Conversely, freezing behavior during the latter extinction retention test represents an index of deficit in fear extinction of the traumatic experience[29]. Our results highlight that the lower is the level of exploratory activity after trauma the higher is the freezing behavior of rats both at the retrieval or extinction sessions (indicative of exaggerated retention and lack of extinction). This result highlights this behavioral parameter as strictly related to the PTSD-like cognitive maladaptive changes of over-consolidation and impaired extinction. One of the overarching features of PTSD in humans, in addition to the cognitive alterations previously mentioned, is social impairment[30]. Several clinical reports indicate social withdrawal and social isolation as common hallmarks of PTSD patients[31–33]. In the animal model that we had previously validated, we found that rats exposed to footshock trauma paired with social isolation displayed reduced social interaction time in the Social Interaction test and impaired memory processes[22]. Here, we found that the exploratory activity of a novel environment soon after trauma not only correlates with cognitive alterations but it also positively correlates with the time spent in social interaction long after trauma exposure. The lower is the exploratory activity the lower is the level of social interaction, thus making this parameter a predictive variable also for the trauma induced maladaptive changes in the emotional domains long after stress exposure. Taken together these results indicate the level of exploratory activity used as a very robust predictive variable to immediately identify subjects that will develop chronic PTSD-like alterations later after trauma. We thus hypothesize that trauma experience enhances the stress responsiveness in susceptible rats to other potentially stressful situation, such as a novel environment and, as a consequence of this, a reduction of their innate tendency for exploration as a form of fear generalization. In support of this hypothesis, we found a different habituation profile in the total distance traveled in the Open Field arena across time among the groups tested before or after trauma. While rats tested before trauma showed a normal habituation profile to the arena with higher explorative activity during

the first phase of the test, traumatized rats did not. Importantly, susceptible rats explored less the Open Field arena for all the test duration, whereas the NRs and resilient ones moved less at the beginning but started to explore more the arena towards the end of the test displaying an opposite habituation profile with respect to rats not exposed to trauma. This phenotype-specific habituation profile has a high translational value and may be ascribable to fear generalization induced by the traumatic experience, which are key features of PTSD and which consist in the transfer of fear to stimuli not related to the aversive event[34–37]. To further test the robustness of the identified predictive variable, we segregated animals in susceptible, normal and resilient, according to the 25th and 75th percentile of the experimental group's distribution for the exploratory activity in the Open Field test. Our results indicate that susceptible rats display increased freezing response compared to resilient rats, across retrieval and extinction sessions, where they show higher level of freezing also with respect to the group exerting normal levels of activity. Thus, resembling the PTSD human clusters, where an over-consolidation of fear memory of the trauma and an impaired extinction learning after exposure therapy sessions are robust endophenotypes of susceptibility to PTSD pathology, whereas, the absence of these maladaptive changes is typical of a resilient phenotype[38]. The same classification revealed significant differences among experimental groups also in the Social Interaction test. Particularly, we found that susceptible rats spent less time interacting with a conspecific after trauma with respect to the resilient ones. These findings add a high translational value to our PTSD animal model with respect to human PTSD. Motor activity has a good translational value since it may resemble the strategies adopted by human subjects to deal with stressful situations, known as coping strategies[39–41]. For a rat the distance traveled in a new arena, as previously mentioned, is a measure of not only motor activity but also of the innate tendency of the animal to explore a new environment moving across it[26]. Literature data indicate a reduction in the motor activity in animals exposed to contextual fear conditioning regardless of their individual susceptibility[42,43]. Prompted by this evidence and results, that we obtained in the first set of experiments, we next aimed to dissociate these two aspects, in order to

better understand if it is the motor activity per se, or the tendency to explore a new environment that made the exploratory behavior after trauma a reliable predictive variable for the development of a PTSD-like phenotype. We thus analyzed pure motor behavior in the rotarod test, but we did not find any significant correlation between the pure motor activity assessed in this test and the behavioral alterations associated with the PTSD-like phenotype, thus excluding the role of the pure motor activity in making the exploratory behavior soon after trauma a predictive variable for the development of a PTSD-like phenotype. We then aimed at clarifying whether the natural tendency to explore an environment regardless of any trauma exposure would be useful to disentangle between resilient and susceptible animals or is it rather the reaction after trauma that, by soon influencing the exploratory behavior, becomes a strong predictive variable to screen between the two phenotypes before the pathology develops. Interestingly, we did not find any significant correlation between the exploratory activity in the Open Field test performed before the traumatic experience and any behavioral outcome associated with the PTSD-like phenotype. These results clearly indicate that only the change induced by the trauma exposure on the exploratory behavior before the pathology occurs make it a predictive variable to soon identify animals susceptible to later develop a PTSD-like phenotype. The Anxiety and Depression Association of America (ADAA)[44] has estimated that physical exercise (e.g. running and walking) and talking to friends are among the most common ways people adopt to cope with stress after trauma. Interestingly, literature data indicate that people with PTSD participate less to physical activity[45–47]. Not only people with PTSD are less physical active, but with respect to people who fully recover after trauma, they also tend to stay at home for longer time and are less interested in exploring new places and in living new experiences, because any kind of stimuli in an unfamiliar environment can trigger suppressed or unwanted memories and emotions[48]. In consideration of the translational value of our present results, it is a tentative to speculate that it could be theoretically possible to detect resilient and susceptible humans' individuals in later developing PTSD, on the basis of their attitude after trauma to soon cope with stress by

practicing physical activity and by their willingness to search for new experiences in unfamiliar environments[45–47]. In conclusion, we here developed a rat animal model able to predict individual differences in later developing a PTSD-like phenotype with a high translational value with respect to the cognitive and emotional clusters observed in the human pathology. Overall, our results pave the road for further studies in which susceptible and resilient animals can be differentially manipulated at the interacting circuit-levels to a better understand the neurobiological underpinnings of susceptibility and resilience towards PTSD development, providing pre-clinical prospects with therapeutic interventions in a rat animal model and, subsequently, translated to humans.

References

1. Berardi, A., Schelling, G. & Campolongo, P. The endocannabinoid system and post traumatic stress disorder (PTSD): from preclinical findings to innovative therapeutic approaches in clinical settings. Pharmacol. Res. 111, 668–678 (2016).
2. Trezza, V. & Campolongo, P. The endocannabinoid system as a possible target to treat both the cognitive and emotional features of post-traumatic stress disorder (PTSD). Front. Behav. Neurosci. 7, 100 (2013).
3. Yehuda, R. et al. Post-traumatic stress disorder. Nat. Rev. Dis. Prim. 1, 15057 (2015).
4. Asok, A., Kandel, E. R. & Rayman, J. B. The neurobiology of fear generalization. Front. Behav. Neurosci. 12, 329 (2018).
5. Herry, C. & Johansen, J. P. Encoding of fear learning and memory in distributed neuronal circuits. Nat. Neurosci. 17, 1644–1654 (2014).
6. Johansen, J. P., Cain, C. K.,Ostroff, L. E. & LeDoux, J. E.Molecular mechanisms of fear learning and memory. Cell 147, 509–524 (2011).
7. Desmedt, A., Marighetto, A. & Piazza, P. V. Abnormal fear memory as a model for posttraumatic stress disorder. Biol. Psychiatry 78, 290–297 (2015).
8. Finsterwald, C., Steinmetz, A. B., Travaglia, A. & Alberini, C. M. From memory impairment to posttraumatic stress disorder-like phenotypes: the critical role of an unpredictable second traumatic experience. J. Neurosci. 35, 15903–15915 (2015).
9. Javidi, H. & Yadollahie, M. Post-traumatic stress disorder. Int. J. Occup. Environ. Med. 3, 2–9 (2012).
10. Lewis, S. J. et al. The epidemiology of trauma and post-traumatic stress disorder in a representative cohort of young people in England and Wales. Lancet Psychiatry 6, 247–256 (2019).
11. McEwen, B. S. Physiology and neurobiology of stress and adaptation: central role of the brain.

Physiol. Rev. 87, 873–904 (2007).
12. Del Giudice, M., Ellis, B. J. & Shirtcliff, E. A. The adaptive calibration model of stress responsivity. Neurosci. Biobehav. Rev. 35, 1562–1592 (2011).
13. Ullmann, E. et al. From allostatic load to allostatic state—an endogenous sympathetic strategy to deal with chronic anxiety and stress? Front. Behav. Neurosci. 13, 47 (2019).
14. Kar, N. Cognitive behavioral therapy for the treatment of post-traumatic stress disorder: a review. Neuropsychiatr. Dis. Treat. 7, 167–181 (2011).
15. Nestler, E. J. & Hyman, S. E. Animal models of neuropsychiatric disorders. Nat. Neurosci. 13, 1161–1169 (2010).
16. Daskalakis, N. P., Yehuda, R. & Diamond, D. M. Animal models in translational studies of PTSD. Psychoneuroendocrinology 38, 1895–1911 (2013).
17. Hendriksen, H., Olivier, B. & Oosting, R. S. From non-pharmacological treatments for post-traumatic stress disorder to novel therapeutic targets. Eur. J. Pharmacol. 732, 139–158 (2014).
18. Sillivan, S. E. et al. Susceptibility and resilience to posttraumatic stress disorderlike behaviors in inbred mice. Biol. Psychiatry 82, 924–933 (2017).
19. Holly, E. N. & Miczek, K. A. Capturing individual differences: challenges in animal models of posttraumatic stress disorder and drug abuse. Biol. Psychiatry 78, 816–818 (2015).
20. Musazzi, L., Tornese, P., Sala, N. & Popoli, M. What acute stress protocols can tell us about PTSD and stress-related neuropsychiatric disorders. Front. Pharmacol. 9, 758 (2018).
21. Ritov, G., Boltyansky, B. & Richter-Levin, G. A novel approach to PTSD modeling in rats reveals alternating patterns of limbic activity in different types of stress reaction. Mol. Psychiatry 21, 630–641 (2016).
22. Berardi, A. et al. An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD). Front. Behav. Neurosci. 8, 142 (2014).
23. Morena, M. et al. Enhancing endocannabinoid neurotransmission augments the efficacy of extinction training and ameliorates traumatic stress-induced behavioral alterations in rats. Neuropsychopharmacology 43, 1284–1296 (2018).
24. Chen, X., Li, Y., Li, S. & Kirouac, G. J. Early fear as a predictor of avoidance in a rat model of post-traumatic stress disorder. Behav. Brain Res. 226, 112–117 (2012).
25. Hadadianpour, Z. et al. The effect of orexin-A on motor and cognitive functions in a rat model of Parkinson's disease. Neurol. Res. 39, 845–851 (2017).
26. Denenberg, V. H. Open-field bheavior in the rat: what does it mean? Ann. NY Acad. Sci. 159, 852–859 (1969).
27. Phillips, R. G. & LeDoux, J. E. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav. Neurosci. 106, 274–285 (1992).
28. Kida, S. Reconsolidation/destabilization, extinction and forgetting of fear memory as therapeutic targets for PTSD. Psychopharmacology 236, 49–57 (2019).
29. Singewald, N. & Holmes, A. Rodent models of impaired fear extinction. Psychopharmacology 236, 21–32 (2019).
30. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders 5th edn (2013).

31. Hofmann, S. G., Litz, B. T. &Weathers, F. W. Social anxiety, depression, and PTSD in Vietnam veterans. J. Anxiety Disord. 17, 573–582 (2003).
32. Kashdan, T. B., Morina, N. & Priebe, S. Post-traumatic stress disorder, social anxiety disorder, and depression in survivors of the Kosovo War: experiential avoidance as a contributor to distress and quality of life. J. Anxiety Disord. 23, 185–196 (2009).
33. LaMotte, A. D., Taft, C. T., Weatherill, R. P. & Eckhardt, C. I. Social skills deficits as a mediator between PTSD symptoms and intimate partner aggression in returning veterans. J. Fam. Psychol. 31, 105–110 (2017).
34. Colucci, P. et al. Amphetamine and the smart drug 3,4-methylenedioxypyrovalerone (MDPV) induce generalization of fear memory in rats. Front. Mol. Neurosci. 12, 292 (2019).
35. Dymond, S., Dunsmoor, J. E., Vervliet, B., Roche, B. & Hermans, D. Fear generalization in humans: systematic review and implications for anxiety disorder research. Behav. Ther. 46, 561–582 (2015).
36. Hammell, A. E., Helwig, N. E., Kaczkurkin, A. N., Sponheim, S. R. & Lissek, S. The temporal course of over-generalized conditioned threat expectancies in posttraumatic stress disorder. Behav. Res. Ther. 124, 103513 (2020).
37. Lis, S. et al. Generalization of fear in post-traumatic stress disorder. Psychophysiology 57, e13422 (2020).
38. Hill, M. N., Campolongo, P., Yehuda, R. & Patel, S. Integrating endocannabinoid signaling and cannabinoids into the biology and treatment of posttraumatic stress disorder. Neuropsychopharmacology 43, 80–102 (2018).
39. Feder, A., Nestler, E. J. & Charney, D. S. Psychobiology and molecular genetics of resilience. Nat. Rev. Neurosci. 10, 446–457 (2009).
40. Koolhaas, J. M., de Boer, S. F., Buwalda, B. & van Reenen, K. Individual variation in coping with stress: a multidimensional approach of ultimate and proximate mechanisms. Brain Behav. Evol. 70, 218–226 (2007).
41. Puglisi-Allegra, S. & Andolina, D. Serotonin and stress coping. Behav. Brain Res. 277, 58–67 (2015).
42. Daviu, N., Fuentes, S., Nadal, R. & Armario, A. A single footshock causes longlasting hypoactivity in unknown environments that is dependent on the development of contextual fear conditioning. Neurobiol. Learn. Mem. 94, 183–190 (2010).
43. Radulovic, J., Kammermeier, J. & Spiess, J. Generalization of fear responses in C57BL/6N mice subjected to one-trial foreground contextual fear conditioning. Behav. Brain Res. 95, 179–189 (1998).
44. Anxiety and Depression Association of America (ADAA). Exercise for Stress and Anxiety. https://adaa.org/living-with-anxiety/managing-anxiety/exercise-stress and anxiety (2011).
45. Rosenbaum, S. et al. Among inpatients, posttraumatic stress disorder symptom severity is negatively associated with time spent walking. J. Nerv. Ment. Dis. 204, 15–19 (2016).
46. Vancampfort, D. et al. Physical activity in people with posttraumatic stress disorder: a systematic review of correlates. J. Phys. Act. Health 13, 910–918 (2016).
47. de Assis, M. A. et al. Evaluation of physical activity habits in patients with posttraumatic

stress disorder. Clinics 63, 473–478 (2008).
48. Johannessen, K. B. & Berntsen, D. Current concerns in involuntary and voluntary autobiographical memories. Conscious. Cogn. 19, 847–860 (2010).

CHAPTER 5

SOCIAL DEFEAT STRESS DURING EARLY ADOLESCENCE CONFERS RESILIENCE AGAINST A SINGLE EPISODE OF PROLOGED STRESS IN ADULT RATS

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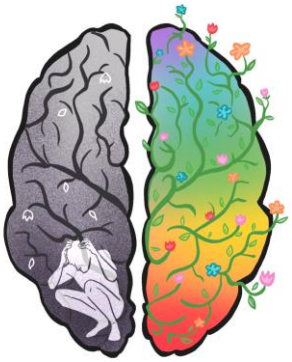

Abstract

Early-life adverse experiences (first hit) lead to coping strategies that may confer resilience or vulnerability to later experienced stressful events (second hit) and the subsequent development of stress-related psychopathologies. Here, we investigated whether exposure to 2 stressors at different stages in life has long-term effects on emotional and cognitive capabilities, and whether the interaction between the 2 stressors influences stress resilience. Male rats were subjected to social defeat stress (SDS, first hit) in adolescence and to a single episode of prolonged stress (SPS, second hit) in adulthood. Behavioral outcomes, hippocampal expression of brain-derived neurotrophic factor, and plasma corticosterone levels were tested in adulthood. Rats exposed to both stressors exhibited resilience against the development of stress-induced alterations in emotionality and spatial memory, but vulnerability to cued fear memory dysfunction. Rats subjected to both stressors demonstrated resilience against the long-term effects of SDS-induced alterations in hippocampal brain-derived neurotrophic factor expression and plasma corticosterone levels. SPS alone altered locomotion and spatial memory retention; these effects were absent in SDS-exposed rats later exposed to SPS. Our findings reveal that exposure to social stress during early adolescence influences the ability to cope with a second challenge experienced later in life.

Introduction

Stress exposure can lead to the development of several psychiatric diseases, including anxiety, depression, and post-traumatic stress disorder (PTSD) [1,2]. Early-life stressful experiences may lead to coping strategies that match or mismatch later-life adverse experiences, resulting in resilience or vulnerability, respectively, to the development of psychopathologies in adulthood [3–6]. Because adolescence is a crucial developmental stage associated with profound changes in the structure and function of the brain [7–9], stress experienced during this critical developmental period has more detrimental effects com-pared with those experienced in adulthood. The hypothalamic–pituitary–adrenal axis is still immature in early adolescence, and the release of cortisol (corticosterone in rodents) in response to acute stressors is higher than that in adulthood [10–12]. Chronic glucocorticoid exposure during adolescence is associated with adverse consequences, particularly in the hippocampus where it causes neuronal cell damage and dendritic atrophy, reduces neurogenesis, impairs synaptic plasticity, and suppresses long-term potentiation [13–16]. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, regulates axonal guidance, enhances synaptic plasticity and neurite outgrowth, and facilitates long-term potentiation [17]. Glucocorticoids strictly regulate the expression and signaling of BDNF, and a growing body of evidence indicates that a stress-induced change in BDNF signaling is a potential cause of stress-related disease [18–21]. Patients with depression or bipolar disorder have altered hippocampal BDNF expression [22], and patients with de-pression or PTSD have reduced hippocampal volume [23,24].

Adolescence is characterized by increased social interactions, as well as play and novelty-seeking behaviors, which are necessary for acquiring the skills related to developing autonomy and independence from parental caretakers [8]. Bullying and subordination episodes are social stressors that frequently occur among adolescents [25,26]. Numerous studies report that adolescent victims of bullying are more predisposed to mental disorders later in life, particularly anxiety and depression [27]. Human data reporting the in-fluence of experiencing bullying during adolescence on

the development of psychiatric disorders and their seriousness after additional trauma in adulthood are lacking. Under-standing how early-life social stress could change an individual's responsiveness to additional trauma later in life will facilitate the development of treatments for stress-related disorders such as PTSD in daily clinical practice.

The social defeat stress (SDS) paradigm is widely used in rodents as a highly vali-dated animal model of bullying. By using the resident–intruder model, which includes social subordination, threat, and physical abuse, it is possible to reproduce and study the main features of bullying in humans [28,29]. Generally, this paradigm consists of placing an experimental rodent (intruder) into the territory of a larger and aggressive conspecific (resident) that attacks the intruder for one or more sessions [30]. Compelling evidence indicates that SDS can have both short- and long-term consequences on emotional and cognitive domains, such as increased anxiety and altered cognitive flexibility, in adult rodents. Exposure to adverse stimuli, particularly during sensitive windows of brain development, is a critically important environmental factor involved in an individual's response to stress [31]. Individuals can be classified into two categories on the basis of their reaction to stress: vulnerable people that negatively respond to adverse stressful stimuli, and resilient people that positively respond to the same stimuli [32,33]. Various hypotheses have been postulated to explain this difference between individuals. According to the cumulative stress theory, individuals that are continuously exposed to stress across their life span are more predisposed to develop psychopathologies [34]. Conversely, in the match/mismatch hypothesis, vulnerability or resilience to stress depends on the interaction between early-life and adult stress. The two-hit stress model has been used to investigate whether exposure to two different stressors at different ages increases or decreases the risk of developing psychopathologies after experiencing the second stressor [35–40]. The effects of social stress similar to bullying in humans experienced during adolescence on the reaction to an additional stressor later in life, however, are less investigated [41].

To elucidate this issue, we exposed rats to SDS during early adolescence and then

exposed them in adulthood to a single prolonged stress (SPS) as a second stressor. SPS is a validated stress paradigm comprising three stressful events presented during a single session [42–44]. We evaluated whether exposure to stress during early adolescence (first hit) affects emotionality and cognitive processes in the long-term, and whether exposure to a second challenge (second hit) later in life alters such effects. Rats were exposed to SDS during early adolescence and to SPS in adulthood, and then subjected to a behavioral test battery, including the open field, acoustic startle response, Morris water maze, and auditory fear conditioning. Further, to study the interplay between stress and BDNF in these effects, we examined hippocampal BDNF protein expression and plasma corticosterone levels in adulthood. Further, we evaluated the long-term effects induced by the two stressors on BDNF levels in the brain region most critically affected by stress effects, i.e., the hippocampus. We then evaluated whether any hippocampal BDNF alterations were paralleled by changes in plasma corticosterone concentrations.

Material and methods

Animal care

Sprague-Dawley rats (Charles River Laboratories, Calco, Italy) were housed in air-conditioned vivarium rooms (temperature: 21 ± 1°C; lights on from 7:00 am to 7:00 pm) with pellet food and water available ad libitum. Adolescent male rats were housed in groups of 4 per cage until postnatal day (PND) 70 when animals of the same treatment were pair-housed. Adult Sprague-Dawley male rats 400-450 g (Charles River Laboratories, Calco, Italy), used as residents for the SDS protocol, were housed with a sterile, but sexually receptive adult Sprague-Dawley female rat to enhance their aggressiveness towards the intruders [45]. All the experiments were performed during the light phase of the cycle (10:00 AM – 3:00 PM) and were in compliance with the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, the D. L. 26/2014 of Italian Ministry of Health and approved by the Italian Ministry of Health.

Social defeat stress

We used a slightly modified SDS protocol previously described [28,46]. Briefly, for 7 consecutive days (PND 28-34), adolescent intruders were placed in the resident's home cage (the female rat was removed for the duration of the encounter). Each session began with a period of investigation and free interaction until 15 min had elapsed. Rats were then separated by a wire mesh barrier (1-cm weave), allowing continued olfactory and visual contact, but preventing physical contact for the duration of the 30-min session. Controls were picked up from the resident's home cage and then returned to their home cage. The resident-intruder paradigm was repeated daily for 7 days with the intruders being exposed to a different resident each day to prevent any habituation to the resident aggressor. Residents that failed to consistently attack or that were overly aggressive and caused injury to intruders were not used.

Single prolonged stress protocol

We used a previously described SPS protocol [43,47,48] with slight modification. Briefly, rats at PND 90 were exposed to a single session of SPS that consisted of 2 h of restraint stress in a restrainer (30 cm in length with an inside diameter of 7.5 cm), 15 min of forced swim stress (24±1 °C water temperature) followed by 15 min of recovery, and then isoflurane exposure until loss of consciousness. Rats not assigned to SPS groups were placed in a separate room throughout the duration of SPS. After the SPS procedure, all rats were returned to their home cage and left undisturbed for 30 days.

Experimental design

As shown in Fig. 1, adolescent rats at PND 28 were randomly assigned to 1 of 4 groups:

- CTRL-CTRL group: rats not exposed to any stressors
- SDS-CTRL group: rats exposed to only SDS during the adolescence (PND 28-34)
- CTRL-SPS group: rats exposed to only SPS at PND 90
- SDS-SPS group: rats exposed to SDS during adolescence and SPS at PND 90

Animals were left undisturbed for 30 days after SPS exposure in order to evaluate

emotional and cognitive effects long-after trauma and then exposed to a behavioral test battery.

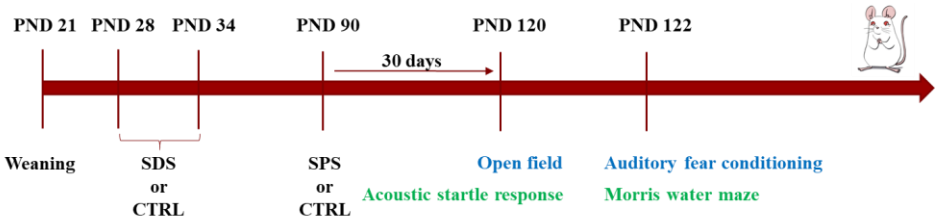

Figure 1: Experimental timeline. Separate cohorts of male rats at PND 28 were randomly assigned to either the CTRL-CTRL, SDS-CTRL, CTRL-SPS, or SDS-SPS groups. Rats were subjected to 30 min SDS daily for 7 consecutive days from PND 28 to 34, and then to the SPS paradigm in adulthood (PND 90). Thirty days after SPS exposure, 2 separate groups of rats were exposed to a behavioral test battery. The first group was exposed to open field (PND 120) and auditory fear conditioning (PND 122) tasks, and the second one was exposed to acoustic startle response (PND 120) and Morris water maze (PND 122) tasks. One week after the last behavioral task, rats were killed, and the bilateral hippocampus was immediately dissected from each animal for biochemical analyses.

Behavioral tests

Open field

In the open field test, each rat was placed into the corner of the open field arena (80 × 80 × 60 cm^3). The test was performed under dim light conditions (2 lux) and lasted 20 min, as previously described [49]. The total distance traveled (cm) by each rat and the time spent in the center of the arena were determined as parameters indicating the rat locomotor activity and emotional reactivity, respectively. After each session, rats were returned to their home cage, fecal boli were removed, and the walls and floor of the arena were cleaned with a 70% ethanol solution. The total distance traveled was acquired and analyzed using an automated video-tracking system (Smart, Panlab, Barcelona, Spain).

Acoustic startle response

The acoustic startle response was measured as previously described [47] with slight modification. Rats were placed in a startle reflex apparatus (Med Associates, Fairfax, VT, USA) for a 5-min acclimatization period with a 70-dB background noise, which continued for the entire the session. Each session consisted of 22 pulse trials (115 db) with intertrial intervals selected randomly between 10 and 15 s. Acoustic devices and startle cages were connected to a computer, which detected and analyzed all chamber variables using customized software (Med Associates). The system allows recording and analysis of the signal generated by the animal movement through a high-sensitivity weight transducer system. The mean startle reflex response for each animal was calculated as the average of the responses to the 22 pulse trials.

Morris water maze

The experimental apparatus was a circular tank (1.83 m in diameter and 0.58 m in height) filled with water (23–24 °C) to a depth of 20 cm. The maze was located in a room containing many salient, visual, extra-maze cues. During the spatial training a rectangular platform (20 x 25 cm^2) was placed at a fixed location 25 cm away from the edge of the pool and 2.5 cm below the water surface. The experiments were performed according to a previously described procedure [50,51]. For spatial training, the rats were given 4 trials on each daily session for 3 consecutive days. On each trial, the animal was placed in the tank facing the wall at one of the 4 designated start positions and allowed to escape onto the hidden platform. If an animal failed to find the platform within 60 s during the first day of the training, it was manually guided to the platform. The rat was allowed to remain on the platform for 10 s and was then placed into a holding cage for 25 s until the start of the next trial. The time each animal spent to reach the platform was recorded as the escape latency. For the acquisition phase, the average of escape latencies across the trials during each day of training was analyzed. Retention of the spatial training was assessed 24 h after the last training session with a 60-s free-swim probe trial and a new starting position was used. Training and probe trials were

videotaped, and an automated tracking system (Smart, Panlab, Barcelona, Spain) was used to analyze the swim path of each subject and calculate the initial latency to cross the platform location and the number of crossings through the platform location. The target and opposite quadrants were equidistant from the starting position on the probe trial.

Auditory fear conditioning

We used a slightly modified protocol as previously described [52,53]. All phases of the auditory fear conditioning task were performed in the same but contextually modified operant chambers, located in sound-attenuating cubicles (Med Associates, Fairfax, VT, USA). The floor of the chambers consisted of stainless-steel bars that delivered a scrambled electric footshock. On day 1, rats were exposed to chamber A and received 5 habituation tones (30 s, 4 kHz, 75 dB; 3 min intertrial interval), immediately followed by 7 conditioning tones that co-terminated with footshocks (0.5 s, 0.65 mA). On day 2, rats were ex-posed to chamber B (contextually modified with black walls) for the extinction session, which consisted of 12 tones (30 s, 4 kHz, 75 dB; 3 min intertrial interval) in the absence of footshock. On day 3, rats were returned to the chamber B and presented with 8 tones (30 s, 4 kHz, 75 dB; 3 min intertrial interval) in the absence of footshock to test for extinction retrieval. Freezing behavior, defined as the absence of all movements except for those related to breathing [54], served as the measurement of fear and was monitored with digital video cameras. An experimenter blinded to the experimental conditions quantified the total time that rats spent freezing during the 30-s tone presentations by using a digital stopwatch. The percentage of time spent in freezing during the 30-s tone presentations was calculated as: [time spent in freezing during the tone presentation (s) / 30 s] x 100. For the conditioning session (day 1), the time spent in freezing during the first and the last tone presentation was represented. For the extinction session (day 2) the average of time spent in freezing during the 3 blocks of 4 trials each was evaluated, while for the retrieval session (day 3) the average of percentage of time spent in freezing during all trials was represented.

Biochemical analyses

Tissue collection and Western blotting analysis

Western blotting analysis was performed as previously described [55,56]. Rats were decapitated after 7 days from the last behavioral task to measure BDNF expression at baseline levels. The hippocampus was rapidly collected, pooled from both side of the brain, and homogenized in lysis buffer, containing Tris-HCl (pH 8) 50mM, NaCl 150 mM, NP40 1%, SDS 0.1% and a protease inhibitor mixture (Sigma, St. Louis, 154 MO, USA, P8340, 1/100) in distilled water. Tissues were centrifuged at 15000 rpm for 10 min, and the supernatant removed and stored in aliquots at -80 °C until use. Equivalent amounts (30 μg) of each sample calculated by Bradford assay were resolved on 12% acrylamide SDS-PAGE gels, and then transferred onto nitrocellulose. Membranes were blocked for 1 h at room temperature in a solution containing 5% non-fat dry milk (Bio Basic, Markham, ON, CA) in tris-buffered saline (TBS) 0.1% tween 20 (TBS-T). Membranes were then incubated overnight at 4 °C with anti BDNF rabbit polyclonal antibody (1:500, Bioss Antibodies, Boston, MA, USA) or anti β-actin goat polyclonal antibody (1:500, Biorbyt Ltd, Cambridge, UK). After being washed 3 times for 10 min in TBS-T, membranes were incubated for 1 h at room temperature in the proper secondary horseradish peroxidase-conjugated antibodies (HRP-conjugated goat anti-rabbit IgG, 1:10000, Thermo Fisher Scientific, Rockford, IL, USA; HRP-conjugated mouse anti-goat IgG, 1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was developed with enhanced chemiluminescence (ECL system; BioRad, CA, USA). For analysis of the Western blotting data, densitometric analysis was performed using Image J software.

Plasma corticosterone levels

Rats were decapitated after 7 days from the last behavioral task in order to measure baseline corticosterone levels. Trunk blood was collected after decapitation (11:30 AM – 13:30 PM) in tubes containing 200 μl of 0.1M EDTA and samples were centrifuged

at 1000 × g for 15 min at 4 °C. Plasma was stored at −20 °C and analyzed for corticosterone levels using a DetectX ELISA kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions, as previously described [57,58].

Statistical Analysis

Statistical analysis was performed using SPSS statistical software. Each measure is expressed as mean ± SEM. A repeated measures ANOVA (RM ANOVA) was used to evaluate the mean escape latency across trials during each training day in the Morris water maze and the percentage of freezing during the conditioning and extinction phases of the auditory fear conditioning task. In all other cases, data were analyzed with 2-way ANOVA when appropriate. Biochemical data were analyzed with a 2-way ANOVA. The Tukey-Kramer post-hoc test was performed to control for significant differences between groups when appropriate. Significance was considered for $P < 0.05$.

Results

The interaction between SDS during early adolescence and SPS in adulthood induces resilience towards the development of hyperarousal and anxiety later in life

Open field test

We evaluated whether the exposure to SDS during early adolescence and/or to SPS in adulthood induced enduring alterations on locomotor activity and emotionality. As shown in Fig. 2a, 2-way ANOVA for the distance traveled in the open field showed no significant effect of SDS ($F_{(1,35)} = 0.205$; $P = 0.653$), a significant effect of SPS ($F_{(1,35)} = 5.291$; $P = 0.028$), and no significant SDS x SPS interaction ($F_{(1,35)} = 2.726$; $P = 0.108$). Post hoc analysis revealed that rats exposed to only SPS traveled shorter distance compared with control rats ($P < 0.05$). Two-way ANOVA showed no significant main effects for the time spent in the center of the open field arena for SDS ($F_{(1,35)} = 0.141$; P

= 0.710) and SPS ($F_{(1,35)}$ = 0.551; P = 0.463), but revealed a significant SDS x SPS interaction ($F_{(1,35)}$ = 4.631; P = 0.038, Fig. 2b). Post hoc analysis revealed that rats exposed to only SDS spent less time in the center of the open field arena compared with control rats (P < 0.05). These results indicate that SDS experienced during early adolescence does not affect locomotor activity in adulthood, but does induce an anxious-like profile in adult rats. Conversely, rats exposed to SPS in adulthood and tested in the open field test exhibited reduced locomotor activity, but no alterations of emotional parameters. Interestingly, rats subjected to both stressors exhibited no alterations in locomotor activity or emotionality, indicating that SDS in adolescence combined with SPS experienced later in life confers resilience to stress.

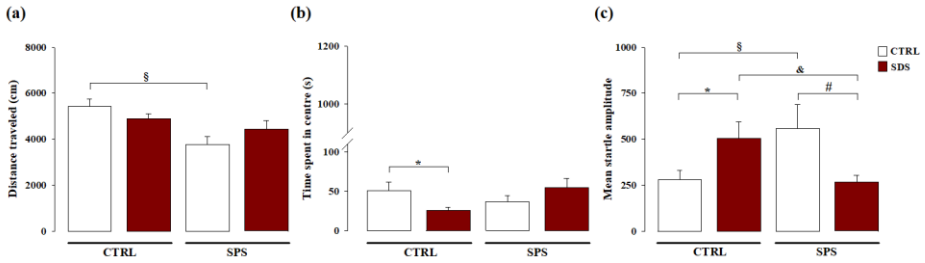

Figure 2: Long-term effects on locomotor activity, hyperarousal, and anxiety-like behavior induced by SDS, SPS, or the combination of both stressors. Long-term effects induced by SDS, SPS, or both stressors on the (a) distance traveled (cm) evaluated in the open field arena, (b) time spent in the center of the arena in the open field, and (c) mean startle amplitude in the acoustic startle response task. *, P < 0.05 vs. CTRL-CTRL group; §, P < 0.05 vs. CTRL-CTRL group; &, P < 0.05 vs. SDS-CTRL group; #, P < 0.05 vs. CTRL-SPS group. (N = 8-12 rats per group or N = 8-11 rats per group, in the open field or acoustic startle response tasks, respectively).

Acoustic startle response

We evaluated whether SDS during early adolescence, SPS in adulthood, or the combination of both stressors may induce long-term effects on hyperarousal and anxiety-like behavior. As shown in Fig. 2c, two-way ANOVA for the mean startle

amplitude showed no significant effect of SDS ($F_{(1,33)} = 0.170$; $P = 0.683$), or SPS ($F_{(1,33)} = 0.064$; $P = 0.801$), but a significant effect of SDS x SPS interaction ($F_{(1,33)} = 11.166$; $P = 0.002$). Post hoc analysis revealed that rats singularly exposed to SDS or to SPS had a higher mean startle amplitude than control rats ($P < 0.05$). Conversely, rats subjected to both stressors showed lower mean startle amplitude than rats only exposed to SDS or to SPS ($P < 0.05$) and similar to that of control rats never exposed to any stressor. These results indicate that rats singularly exposed to SDS during early adolescence or to SPS in adulthood and tested in the startle apparatus show hyperarousal and increased anxiety later in life and that such effects are absent when rats are subjected to both stressors, thus demonstrating again that SDS during early adolescence together with the second challenge experienced later in life promotes resilience towards emotional alterations.

The interaction between SDS during early adolescence and SPS in adulthood induces resilience towards spatial memory deficits later in life

We examined whether SDS during early adolescence and/or SPS in adulthood may alter spatial memory in the long-term and whether these effects are altered when rats experienced both stressors in the Morris water maze task. As shown in Fig. 3a, RM ANOVA for escape latency during spatial training revealed a significant effect of trials ($F_{(2,66)} = 86.483$; $P < 0.0001$), indicating that all groups progressively learned to locate the platform across the 3 training days (no other statistical differences were found). For retention memory, during the probe trial 2-way ANOVA for the initial latency to cross the platform location indicated a significant main effect of SDS ($F(_{1,33)} = 7.065$; $P = 0.012$), no significant effect of SPS ($F_{1,33)} = 2.248$; $P = 0.143$), but a significant interaction between these 2 factors ($F_{1,33)} = 6.279$; $P = 0.017$; Fig. 3b). Post hoc analysis revealed that the latency to cross the platform location of rats exposed to only SPS was higher than that of the control group ($P < 0.05$) and those of rats subjected to both stressors ($P < 0.01$). Two-way ANOVA for the number of crossings through the platform location revealed no significant effect of SDS ($F_{1,33)} = 2.260$; $P = 0.142$), a

significant main effect of SPS ($F_{1,33)}$ = 4.061; P = 0.052) and no significant effect of SDS x SPS interaction ($F_{1,33)}$ = 1.790; P = 0.190; Fig. 3c). Post hoc analysis indicated that the crossings through the platform location of rats only exposed to SPS were lower than those of controls (P < 0.05) and those of rats subjected to both stressors (P < 0.05). These results demonstrate that rats singularly exposed to SPS had spatial memory retention deficits. Rats that experienced both SDS during early adolescence and SPS in adulthood did not show these cognitive deficits.

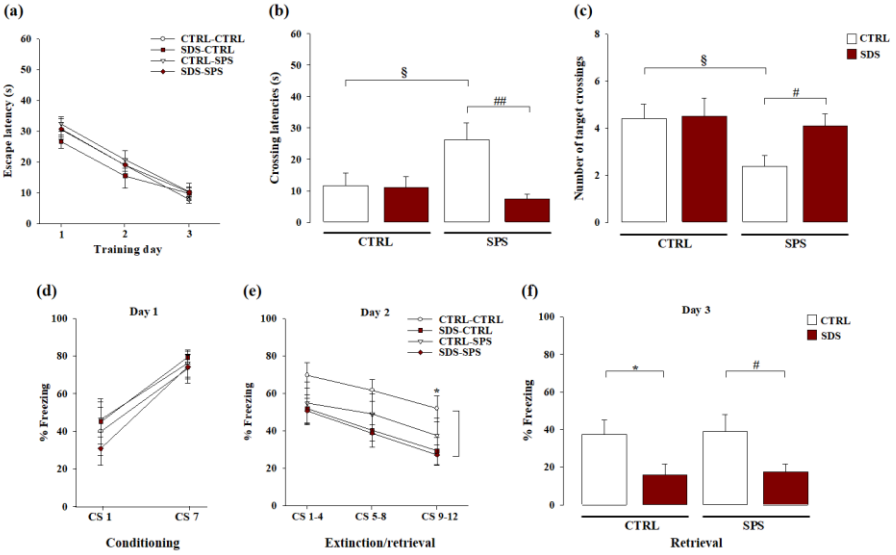

Figure 3: Long-term effects on spatial and cued fear memory dynamics induced by SDS, SPS, or the combination of both stressors. Long-term effects induced by SDS, SPS, or both stressors in the Morris water maze on (a) escape latencies across trials during each day of the acquisition phase, (b) the latency to cross the platform location, and (c) the number of crossings over the platform location during the probe session. Long-term effects induced by SDS, SPS, or both stressors in the auditory fear conditioning paradigm on (d) percentage of time spent in freezing during the first and last tone presentations at conditioning (day 1), (e) mean percentage of time spent in freezing during the 3 blocks of 4 trials each at extinction (day 2), (f) mean percentage of time spent freezing during all 8 trials at extinction retrieval (day 3). *, P < 0.05 vs. CTRL-CTRL group; §, P < 0.05 vs. CTRL-CTRL group; #, P < 0.05 vs. CTRL-SPS group; ##,

$P < 0.01$ vs. CTRL-SPS group. (N = 8-11 rats per group or N = 8-12 rats per group, in the Morris water maze or auditory fear conditioning tasks, respectively).

The interaction between SDS during early adolescence and SPS in adulthood induces vulnerability towards cued fear memory deficits later in life

We evaluated whether SDS during early adolescence and/or SPS in adulthood alter cued fear memory dynamics in adulthood and whether the exposure to both stressors may affect these effects in the auditory fear conditioning task. As shown in Figure 3d, RM ANOVA for the percentage of freezing during fear conditioning acquisition (day 1) revealed a significant main effect of tone presentation ($F_{(1,35)} = 42.958$; $P < 0.0001$), indicating that all groups learned to associate the tone with the footshock (no other statistical differences were found). RM ANOVA for the time spent in freezing during the memory extinction phase on day 2 (Figure 3e) revealed significant main effects of tone presentation ($F_{(1,35)} = 14.625$; $P < 0.0001$) and SDS ($F_{(1,35)} = 4.692$; $P = 0.037$), but no significant main effect of SPS ($F_{(1,35)} = 1.389$; $P = 0.247$) or SDS x SPS interaction ($F_{(1,35)} = 0.885$; $P = 0.353$). No other statis-tical differences were found. Post hoc test revealed that rats only exposed to SDS froze less than controls during the last 4 tones presentation ($P < 0.05$). Two-way ANOVA for the time spent in freezing during the memory retrieval session on day 3 (Figure 3f), showed a significant main effect of SDS ($F_{(1,35)} = 10.089$; $P = 0.003$), but no significant main effect of SPS ($F_{(1,35)} = 0.055$; $P = 0.817$) or SDS x SPS interaction ($F_{(1,35)} = 0.0001$; $P = 0.991$). Post hoc analysis showed that rats only exposed to SDS and rats exposed to both stressors presented less mean percentage of freezing as compared with control rats and rats exposed to only SPS, respectively ($P < 0.05$). These results indicate that exposure to SDS during early adolescence altered extinction and extinction retrieval in adult rats, while SPS did not. Further, when rats were exposed to both stressors fear memory dysfunctions induced by SDS persist, thus indicating that SDS combined with SPS produces vulnerability towards fear memory dynamics.

The interaction between SDS during early adolescence and SPS in adulthood normalizes protein levels of BDNF within the hippocampus later in life

We examined whether exposure to SDS during early adolescence and/or SPS in adulthood modulates BDNF protein expression levels within the hippocampus in adulthood. We performed Western blot analysis to compare BDNF protein expression levels within the hippocampus of all 4 groups of tested rats. As shown in Fig. 4a, 2-way ANOVA for hippocampal BDNF levels showed significant effects of SDS ($F_{(1,28)}$ = 11.074; P = 0.003), SPS ($F_{(1,28)}$ = 15.960; P < 0.001) and interaction between these 2 factors ($F_{(1,28)}$ = 10.124; P = 0.004). Post hoc analysis revealed that rats exposed to only SDS have increased hippocampal BDNF protein expression compared with control rats (P < 0.01) and rats exposed to both stressors (P < 0.01). These results indicate that exposure to SDS during early adolescence induced an increase in BDNF protein expression in the hippocampus of adult rats, while SPS did not. Moreover, these alterations induced by SDS were absent in rats exposed to both stressors. The representative Western blot bands are shown in Fig. 4b.

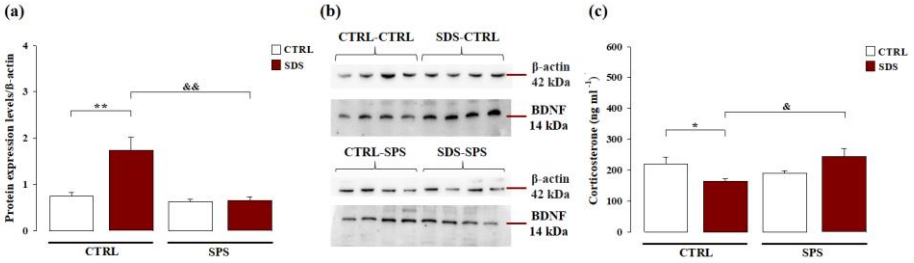

Figure 4: Long-term effects on hippocampal BDNF protein expression and plasma corticosterone levels in rats exposed to SDS, SPS, or the combination of both stressors. (a) Western blotting analysis of hippocampal BDNF protein expression levels in adult rats. Data represent means (±SEM) from 3 replicate experiments and are normalized with β-actin and (b) representative Western blot bands of 4 samples. (c) Plasma corticosterone levels. Data represent means (±SEM) from 3 replicate experiments. *, P < 0.05 vs. CTRL-CTRL group; **, P < 0.01 vs. CTRL-CTRL group; &, P < 0.01 vs. SDS-CTRL group; &&, P < 0.01 vs. SDS-CTRL group. (N = 6-8 per group).

The interaction between SDS during early adolescence and SPS in adulthood normalizes the corticosterone plasma levels later in life

Long-term effects of the exposure to SDS during early adolescence and/or SPS in adulthood on corticosterone plasma levels were evaluated. As shown in Fig. 4c, 2-way ANOVA for plasma corticosterone levels did not reveal a significant main effect of SDS ($F_{(1,20)} < 0.001$; $P = 0.988$) or SPS ($F_{(1,20)} = 2.017$; $P = 0.171$), but a significant interaction between these 2 factors ($F_{(1,20)} = 9.889$; $P = 0.005$). Post hoc analysis revealed that rats exposed to only SDS had lower corticosterone plasma levels than control rats ($P < 0.05$) and rats subjected to both stressors ($P < 0.05$). These results indicate that exposure to SDS during early adolescence, but not to SPS in adulthood or to both stressors, induced low plasma corticosterone levels in adult rats.

Discussion

The present findings indicate that exposure to social stress during early adolescence influences the ability to cope with a second challenge experienced later in life. Specifically, rats exposed to SDS during early adolescence and then to SPS in adulthood exhibited resilience against the development of alterations in emotionality, arousal, and spatial memory, but demonstrated vulnerability toward cued fear memory dysfunction. In addition, rats exposed to both stressors showed resilience toward alterations induced by SDS exposure on the hippocampal BDNF protein expression and plasma corticosterone levels. These effects seemed to be bidirectional; SPS alone induced alterations of locomotor activity and spatial memory retention that were not detected in rats that experienced SDS during adolescence.

SDS exposure affects both the emotional and cognitive domains in rodents, and these effects are profound and enduring when the social stress occurs during important developmental stages [59,60]. SDS is a validated experimental model that mimics some of the effects of bullying victimization in humans [28,29], a stressor that frequently occurs among school-age adolescents. Adolescent victims of bullying are characterized by an enhanced risk for developing mental disorders later in life, such as anxiety,

depression, and PTSD [27,61]. Not all adolescents experiencing stressful situations, however, develop psychiatric diseases in adulthood [62,63]. According to the match/mismatch hypothesis, vulnerability or resilience to developing a psychopathology in adulthood depends by how early-life stressful events match the adversities in later life [3–6]. Here, by using the resident–intruder paradigm (SDS) as a first stressor and an acute traumatic stress (SPS) as the second stressor, we assessed whether the interaction between SDS during early adolescence and SPS in adulthood alters the vulnerability or resilience towards long-term emotional and cognitive function. In accordance with previous studies [59,60], we found that adolescent rats exposed to chronic social defeat for seven days developed long-term emotional impairments in adulthood, as indicated by a reduced time spent in the center of the open field arena and an enhanced startle response. Moreover, SDS during adolescence was linked to distinct patterns of cognitive impairment. We found impairments in cued fear memory, while spatial memory was not affected. Cued fear conditioning is widely used to measure emotional learning and memory [64-66]. In this behavioral paradigm, animals learn to relate an aversive event, such as a footshock, with a stimulus, such as a tone, and subsequently express fear responses after stimulus presentations. Consistent with our results indicating that SDS during early adolescence altered cued fear memory alterations in the long-term, Novick and colleagues reported decreased freezing in rats exposed to SDS during adolescence and tested in the fear conditioning task in adulthood, indicating impaired fear learning [67]. In particular, our results demonstrated that fear memory alterations are referred to the extinction phase and not to the consolidation one, as indicated by the lower freezing percentage during the last tones presentation exhibited by adult rats exposed to SDS in adolescence with respect to controls. Interestingly, we found that adult rats subjected to SDS during early adolescence did not differ from controls in locomotor activity in a novel environment. Consistent with our finding, previous evidence demonstrated that subjecting juvenile rats to SDS does not alter locomotor activity in the open field in adulthood [68]. In contrast to previous studies demonstrating that SDS in adult rats impairs spatial memory

function [69-71], we found that exposure to SDS during early adolescence did not produce long-term effects on spatial memory, suggesting that some of the effects of SDS exposure may be an acute response to stress and are not long-lasting.

The SPS paradigm is a well-validated rodent model used to reproduce some of the hallmark symptoms observed in PTSD patients (e.g., hyperarousal, anxiety, and spatial and fear memory deficits) [72-75]. The manifestation of a PTSD-like phenotype following SPS exposure is time-dependent and requires 7–14 days of incubation to develop [44,76,77]. Because PTSD is a chronic debilitating psychiatric disease, the majority of pre-clinical studies that use SPS as a rodent model of PTSD do not evaluate whether behavior-al alterations persist long after trauma [72-75]. In the present work, rats were exposed to SPS at PND 90 and the behavioral outcomes were evaluated at 1 month after trauma exposure. As we previously demonstrated, SPS exposure induced reduced locomotor activity, hyperarousal, anxiety-like behavior, and spatial memory retention deficits long after exposure to a stressful condition [48]. This is consistent with findings from a previous study demonstrating a sustained reduction in exploratory behavior after exposure to stress [49] and with clinical data indicating an exaggerated startle response as a characteristic trait of PTSD patients [78,79]. Hyperarousal is a state of excessive vigilance to-wards different stimuli, and although the real danger may not be present, PTSD patients act as if it is, thus causing distress and impaired social abilities [80]. It is important to note that these SPS-induced effects did not persist when rats were previously exposed to SDS during early adolescence, thus indicating a positive interaction between the two stressors with the social stress experienced during adolescence altering the ability of the rats to cope with additional trauma in adulthood, and thereby leading to increased resilience against stress-induced alterations.

Studies using animal models of early-life stress demonstrate that an imbalance between stress mediators leads to emotional and cognitive impairments [81]. Adolescence is a period of impressive brain maturation in which the structure of the brain is ever-changing [8]. Thus, maintaining a correct balance between mediators that sustain synaptic plasticity is critical. In the present work, we found that SDS experienced during

early adolescence enhances protein expression of hippocampal BDNF and reduces corticosterone plasma levels with respect to control animals, and that these effects are abolished by a combination of SDS and SPS. Exposure to SDS or chronic mild stress increases BDNF expression in young rats, but not in adult rats, who exhibit decreased hippocampal BDNF expression [82,83]. No data are available on the enduring effects induced by SDS on BDNF expression and, to the best of our knowledge, this is the first evidence demonstrating that adult rats exposed to SDS during early adolescence, but not in adulthood, exhibit increased expression of BDNF in the hippocampus that is linked to behavioral alterations (e.g., hyperarousal, anxiety, and cued fear memory deficits). Similar to our results, previous studies showed increased hippocampal expression of polysialylated neuronal cell adhesion molecule, an important key plasticity molecule [84,85] in adult rats previously exposed to peripubertal [86] or juvenile stress [87], indicating that exposure to early-life stress may disrupt hippocampal maturation. BDNF is one of the most important neurotrophic factors thus playing an important role in the modulation of neuronal morphology and synaptic plasticity, particularly within the hippocampus [88]. Previous evidence indicated that hippocampal BDNF expression reaches its highest level during specific critical windows for brain development, such as early-adolescence [89,90]. Of note, BDNF is strictly involved in the regulation of other neurobiological mediators (e.g., endocannabinoids, glucocorticoids) [91,92] which in turn modulate several brain functions. For all these reasons and on the basis of the present finding we hypothesize that altered hippocampal BDNF expression may represent one possible mechanism responsible for the long-term behavioral alterations induced by SDS, suggesting that exposure to social stress during early adolescence may affect hippocampal maturation processes, leading to in-creased hippocampal BDNF levels later in life. Glucocorticoids play an important role in the coping mechanisms in response to stress and modulate BDNF expression in stress-sensitive brain areas such as the hippocampus [18,20]. Moreover, glucocorticoids and BDNF modulate synaptic plasticity mechanisms by inducing opposing effects [17,20]. Here we found that the increased BDNF levels in

adult rats exposed to SDS in early adolescence are paralleled by lower corticosterone plasma levels compared with non-stressed animals or rats exposed to both stressors.

Our results have strong translational value because clinical studies demonstrate that patients with PTSD [93] or depression [94] who experienced childhood trauma have low baseline levels of cortisol. In conclusion, our data indicate that exposure to SDS during early adolescence or to SPS in adulthood experience different effects later in life. In adult rats exposed to both SDS and SPS, the later development of emotional and cognitive alterations strongly depends on the interaction between the early-life challenge and a second challenge later in life. Our study provides important information about a possible mechanism involved in stress-resilience and susceptibility as a consequence of a combination of two stressors at different periods of life. Understanding the neurobiological underpinnings of stress-induced alterations in animal models is extremely relevant in terms of translational value since it may open the way to the identification of new potential pharmacological interventions to treat stress-related disorders and notably to prevent the development of these diseases after experiencing a second trauma later in life. To the best of our knowledge, this study is the first to evaluate whether exposure to an early-life stress protocol that mimics bullying in adolescents affects the development of psychopathologies in adulthood in response to a trauma experienced later in life.

References

1. McEwen, B.S. Mood disorders and allostatic load. Biol. Psychiatry 2003, 54, 200–207.
2. De Kloet, E.R.; Joëls, M.; Holsboer, F. Stress and the brain: From adaptation to disease. Nat. Rev. Neurosci. 2005, 6, 463–475.
3. Santarelli, S.; Lesuis, S.L.; Wang, X.D.; Wagner, K.V.; Hartmann, J.; Labermaier, C.; Scharf, S.H.; Müller, M.B.; Holsboer, F.; Schmidt, M.V. Evidence supporting the match/mismatch hypothesis of psychiatric disorders. Eur. Neuropsychopharmacol. 2014, 24, 907–918, doi:10.1016/j.euroneuro.2014.02.002.
4. Champagne, D.L.; Ronald de Kloet, E.; Joëls, M. Fundamental aspects of the impact of glucocorticoids on the (immature) brain. Semin. Fetal Neonatal Med. 2009, 14, 136–142, doi:10.1016/j.siny.2008.11.006.
5. Krugers, H.J.; Arp, J.M.; Xiong, H.; Kanatsou, S.; Lesuis, S.L.; Korosi, A.; Joels, M.;

Lucassen, P.J. Early life adversity: Lasting consequences for emotional learning. Neurobiol. Stress 2017, 6, 14–21.
6. Daskalakis, N.P.; Bagot, R.C.; Parker, K.J.; Vinkers, C.H.; de Kloet, E.R. The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome. Psychoneuroendocrinology 2013, 38, 1858–1873, doi:10.1016/j.psyneuen.2013.06.008.
7. Casey, B.J.; Jones, R.M.; Hare, T.A. The adolescent brain. Ann. N. Y. Acad. Sci. 2008, 1124, 111–126.
8. Spear , L.P. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev. 2000, doi:10.1016/S0149-7634(00)00014-2.
9. Romeo, R.D.; McEwen, B.S. Stress and the adolescent brain. Ann. N. Y. Acad. Sci. 2006, 1094, 202–214.
10. McCormick, C.M.; Mathews, I.Z.; Thomas, C.; Waters, P. Investigations of HPA function and the enduring consequences of stressors in adolescence in animal models. Brain Cogn. 2010, 72, 73–85.
11. Goldman, L.; Winget, C.; Hollingshead, G.W.; Levine, S. Postweaning development of negative feedback in the pituitary adrenal system of the rat. Neuroendocrinology 1973, 12, 199–211, doi:10.1159/000122169.
12. Andersen, S.L. Trajectories of brain development: Point of vulnerability or window of opportunity? Neurosci. Biobehav. Rev. 2003, 27, 3–18.
13. Haynes, L.E.; Barber, D.; Mitchell, I.J. Chronic antidepressant medication attenuates dexamethasone-induced neuronal death and sublethal neuronal damage in the hippocampus and striatum. Brain Res. 2004, doi:10.1016/j.brainres.2004.05.117.
14. Schoenfeld, T.J.; Gould, E. Stress, stress hormones, and adult neurogenesis. Exp. Neurol. 2012, 233, 12–21.
15. Pavlides, C.; Watanabe, Y.; McEwen, B.S. Effects of glucocorticoids on hippocampal long-term potentiation. Hippocampus 1993, doi:10.1002/hipo.450030210.
16. Pavlides, C.; Ogawa, S.; Kimura, A.; McEwen, B.S. Role of adrenal steroid mineralocorticoid and glucocorticoid receptors in long-term potentiation in the CA1 field of hippocampal slices. Brain Res. 1996, doi:10.1016/S0006-8993(96)00776-7.
17. Jeanneteau, F.; Chao, M.V. Are BDNF and glucocorticoid activities calibrated? Neuroscience 2013, 239, 173–195.
18. Notaras, M.; van den Buuse, M. Neurobiology of BDNF in fear memory, sensitivity to stress, and stress-related disorders. Mol. Psychiatry 2020, 25, 2251–2274.
19. Martinowich, K.; Manji, H.; Lu, B. New insights into BDNF function in depression and anxiety. Nat. Neurosci. 2007, 10, 1089–1093.
20. Suri, D.; Vaidya, V.A. Glucocorticoid regulation of brain-derived neurotrophic factor: Relevance to hippocampal structural and functional plasticity. Neuroscience 2013, 10, 1089–1093.
21. Gray, J.D.; Milner, T.A.; McEwen, B.S. Dynamic plasticity: The role of glucocorticoids, brain-derived neurotrophic factor and other trophic factors. Neuroscience 2013, 239, 214–

227.
22. Autry, A.E.; Monteggia, L.M. Brain-derived neurotrophic factor and neuropsychiatric disorders. Pharmacol. Rev. 2012, 64, 238–258.
23. Bremner, J.D.; Narayan, M.; Anderson, E.R.; Staib, L.H.; Miller, H.L.; Charney, D.S. Hippocampal volume reduction in major depression. Am. J. Psychiatry 2000, doi:10.1176/ajp.157.1.115.
24. Bremner, J.D.; Randall, P.; Vermetten, E.; Staib, L.; Bronen, R.A.; Mazure, C.; Capelli, S.; McCarthy, G.; Innis, R.B.; Charney, D.S. Magnetic resonance imaging-based measurement of hippocampal volume in posttraumatic stress disorder related to childhood physical and sexual abuse—A preliminary report. Biol. Psychiatry 1997, doi:10.1016/S0006-3223(96)00162-X.
25. Rettew, D.C.; Pawlowski, S. Bullying. Child. Adolesc. Psychiatr. Clin. N. Am. 2016, 25, 235–242.
26. Menesini, E.; Salmivalli, C. Bullying in schools: The state of knowledge and effective interventions. Psychol. Heal. Med. 2017, doi:10.1080/13548506.2017.1279740.
27. Gladstone , G.L.; Parker, G.B.; Malhi, G.S. Do Bullied Children Become Anxious and Depressed Adults? J. Nerv. Ment. Dis. 2006, 194, 201–208, doi:10.1097/01.nmd.0000202491.99719.c3.
28. Miczek, K.A. A new test for aggression in rats without aversive stimulation: Differential effects of d-amphetamine and co-caine. Psychopharmacology (Berl.) 1979, 60, 253–259, doi:10.1007/BF00426664.
29. Björkqvist, K. Social defeat as a stressor in humans. Physiol. Behav. 2001, 73, 435–442, doi:10.1016/S0031-9384(01)00490-5.
30. Golden, S.A.; Covington, H.E.; Berton, O.; Russo, S.J. A standardized protocol for repeated social defeat stress in mice. Nat. Protoc. 2011, 6, 1183–1191, doi:10.1038/nprot.2011.361.
31. Andolina, D.; Puglisi-Allegra, S.; Ventura, R. Strain-dependent differences in corticolimbic processing of aversive or re-warding stimuli. Front. Syst. Neurosci. 2015, 8, doi:10.3389/fnsys.2014.00207.
32. Daskalakis, N.P.; Binder, E.B. Schizophrenia in the Spectrum of Gene-Stress Interactions: The FKBP5 Example. Schizophr. Bull. 2015, 41, 323–329, doi:10.1093/schbul/sbu189.
33. McEwen, B.S. In pursuit of resilience: Stress, epigenetics, and brain plasticity. Ann. N. Y. Acad. Sci. 2016, doi:10.1111/nyas.13020.
34. McEWEN, B.S. Stress, Adaptation, and Disease: Allostasis and Allostatic Load. Ann. N. Y. Acad. Sci. 1998, 840, 33–44, doi:10.1111/j.1749-6632.1998.tb09546.x.
35. Corniquel, M.B.; Koenigsberg, H.W.; Likhtik, E. Toward an animal model of borderline personality disorder. Psychophar-macology (Berl.) 2019, 236, 2485–2500.
36. Faraji, J.; Soltanpour, N.; Ambeskovic, M.; Zucchi, F.C.R.; Beaumier, P.; Kovalchuk, I.; Metz, G.A.S. Evidence for ancestral programming of resilience in a two-hit stress model. Front. Behav. Neurosci. 2017, 11, doi:10.3389/fnbeh.2017.00089.
37. Hill, R.A.; Kiss Von Soly, S.; Ratnayake, U.; Klug, M.; Binder, M.D.; Hannan, A.J.; van

den Buuse, M. Long-term effects of combined neonatal and adolescent stress on brain-derived neurotrophic factor and dopamine receptor expression in the rat forebrain. Biochim. Biophys. Acta Mol. Basis Dis. 2014, 1842, 2126–2135, doi:10.1016/j.bbadis.2014.08.009.

38. Peña, C.J.; Kronman, H.G.; Walker, D.M.; Cates, H.M.; Bagot, R.C.; Purushothaman, I.; Issler, O.; Eddie Loh, Y.H.; Leong, T.; Kiraly, D.D.; et al. Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. Science (80-.) 2017, 356, 1185–1188, doi:10.1126/science.aan4491.
39. Tsoory, M.; Cohen, H.; Richter-Levin, G. Juvenile stress induces a predisposition to either anxiety or depressive-like symp-toms following stress in adulthood. Eur. Neuropsychopharmacol. 2007, doi:10.1016/j.euroneuro.2006.06.007.
40. Zalosnik, M.I.; Pollano, A.; Trujillo, V.; Suárez, M.M.; Durando, P.E. Effect of maternal separation and chronic stress on hip-pocampal-dependent memory in young adult rats: Evidence for the match-mismatch hypothesis. Stress 2014, doi:10.3109/10253890.2014.936005.
41. Buwalda, B.; Stubbendorff, C.; Zickert, N.; Koolhaas, J.M. Adolescent social stress does not necessarily lead to a compro-mised adaptive capacity during adulthood: A study on the consequences of social stress in rats. Neuroscience 2013, doi:10.1016/j.neuroscience.2012.12.050.
42. Kohda, K.; Harada, K.; Kato, K.; Hoshino, A.; Motohashi, J.; Yamaji, T.; Morinobu, S.; Matsuoka, N.; Kato, N. Glucocorticoid receptor activation is involved in producing abnormal phenotypes of single-prolonged stress rats: A putative post-traumatic stress disorder model. Neuroscience 2007, 148, 22–33, doi:10.1016/j.neuroscience.2007.05.041.
43. Liberzon, I.; Krstov, M.; Young, E.A. Stress-restress: Effects on ACTH and fast feedback. Psychoneuroendocrinology 1997, 22, 443–453, doi:10.1016/S0306-4530(97)00044-9.
44. Liberzon , I.; López, J.F.; Flagel, S.B.; Vázquez, D.M.; Young, E.A. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: Relevance to post-traumatic stress disorder. J. Neuroendocrinol. 1999, doi:10.1046/j.1365-2826.1999.00288.x.
45. Mümüşoğlu, S.; Hacıvelioğlu, S.; Sökmensüer, L.K.; Karataylı, R.; Süzer, A.; Kaymaz, F. The comparison of the degree of apoptosis in ovaries and fallopian tubes between two different surgical interventions for tubal ligation: A rat model. J. Turkish Ger. Gynecol. Assoc. 2018, 19, 11–16, doi:10.4274/jtgga.2016.0192.
46. Bingham, B.; McFadden, K.; Zhang, X.; Bhatnagar, S.; Beck, S.; Valentino, R. Early adolescence as a critical window during which social stress distinctly alters behavior and brain norepinephrine activity. Neuropsychopharmacology 2011, 36, 896–909, doi:10.1038/npp.2010.229.
47. Ganon-Elazar, E.; Akirav, I. Cannabinoids prevent the development of behavioral and endocrine alterations in a rat model of intense stress. Neuropsychopharmacology 2012, 37, 456–466, doi:10.1038/npp.2011.204.
48. Mancini, G.F.; Marchetta, E.; Riccardi, E.; Trezza, V.; Morena, M.; Campolongo, P. Sex-

divergent long-term effects of single prolonged stress in adult rats. Behav. Brain Res. 2020, 113096, doi:10.1016/j.bbr.2020.113096.
49. Colucci, P.; Marchetta, E.; Mancini, G.F.; Alva, P.; Chiarotti, F.; Hasan, M.T.; Campolongo, P. Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD). Transl. Psychiatry 2020, 10, doi:10.1038/s41398-020-00929-9.
50. Scuderi, C.; Stecca, C.; Valenza, M.; Ratano, P.; Bronzuoli, M.R.; Bartoli, S.; Steardo, L.; Pompili, E.; Fumagalli, L.; Campo-longo, P.; et al. Palmitoylethanolamide controls reactive gliosis and exerts neuroprotective functions in a rat model of Alz-heimer's disease. Cell Death Dis. 2014, 5, doi:10.1038/cddis.2014.376.
51. Morena, M.; De Castro, V.; Gray, J.M.; Palmery, M.; Trezza, V.; Roozendaal, B.; Hill, M.N.; Campolongo, P. Train-ing-associated emotional arousal shapes endocannabinoid modulation of spatial memory retrieval in rats. J. Neurosci. 2015, 35, 13962–13974, doi:10.1523/JNEUROSCI.1983-15.2015.
52. Rodriguez-Romaguera, J.; Sotres-Bayon, F.; Mueller, D.; Quirk, G.J. Systemic Propranolol Acts Centrally to Reduce Condi-tioned Fear in Rats Without Impairing Extinction. Biol. Psychiatry 2009, 65, 887–892, doi:10.1016/j.biopsych.2009.01.009.
53. Atsak, P.; Hauer, D.; Campolongo, P.; Schelling, G.; Fornari, R.V.; Roozendaal, B. Endocannabinoid Signaling within the Basolateral Amygdala Integrates Multiple Stress Hormone Effects on Memory Consolidation. Neuropsychopharmacology 2015, 40, 1485–1494, doi:10.1038/npp.2014.334.
54. Blanchard, D.C.; Blanchard, R.J. Innate and conditioned reactions to threat in rats with amygdaloid lesions. J. Comp. Physiol. Psychol. 1972, 81, 281–290, doi:10.1037/h0033521.
55. Ratano, P.; Petrella, C.; Forti, F.; Passeri, P.P.; Morena, M.; Palmery, M.; Trezza, V.; Severini, C.; Campolongo, P. Pharmaco-logical inhibition of 2-arachidonoilglycerol hydrolysis enhances memory consolidation in rats through CB2 receptor acti-vation and mTOR signaling modulation. Neuropharmacology 2018, 138, 210–218, doi:10.1016/j.neuropharm.2018.05.030.
56. Kanoski, S.E.; Meisel, R.L.; Mullins, A.J.; Davidson, T.L. The effects of energy-rich diets on discrimination reversal learning and on BDNF in the hippocampus and prefrontal cortex of the rat. Behav. Brain Res. 2007, doi:10.1016/j.bbr.2007.05.004.
57. Santori, A.; Colucci, P.; Mancini, G.F.; Morena, M.; Palmery, M.; Trezza, V.; Puglisi-Allegra, S.; Hill, M.N.; Campolongo, P. Anandamide modulation of circadian- and stress-dependent effects on rat short-term memory. Psychoneuroendocrinology 2019, doi:10.1016/j.psyneuen.2019.06.018.
58. Fletcher, K.; Xiong, Y.; Fletcher, E.; Gustafsson, L. Glucocorticoid response to both predictable and unpredictable challenges detected as corticosterone metabolites in collared flycatcher droppings. PLoS ONE 2018, doi:10.1371/journal.pone.0209289.
59. McCormick, C.M.; Green, M.R. From the stressed adolescent to the anxious and depressed adult: Investigations in rodent models. Neuroscience 2013, 249, 242–257.

60. Buwalda, B.; Geerdink, M.; Vidal, J.; Koolhaas, J.M. Social behavior and social stress in adolescence: A focus on animal mod-els. Neurosci. Biobehav. Rev. 2011, 35, 1713–1721.
61. Mukherjee, S.; Clouston, S.; Bromet, E.; Leibowitz, G.S.; Scott, S.B.; Bernard, K.; Kotov, R.; Luft, B. Past Experiences of Getting Bullied and Assaulted and Posttraumatic Stress Disorder (PTSD) after a Severe Traumatic Event in Adulthood: A Study of World Trade Center (WTC) Responders. J. Aggress. Maltreatment Trauma 2020, doi:10.1080/10926771.2018.1555873.
62. Schmidt, M.V. Animal models for depression and the mismatch hypothesis of disease. Psychoneuroendocrinology 2011, doi:10.1016/j.psyneuen.2010.07.001.
63. Ellis, B.J.; Boyce, W.T.; Belsky, J.; Bakermans-Kranenburg, M.J.; Van Ijzendoorn, M.H. Differential susceptibility to the envi-ronment: An evolutionary- neurodevelopmental theory. Dev. Psychopathol. 2011, doi:10.1017/S0954579410000611.
64. Selcher, J.C.; Nekrasova, T.; Paylor, R.; Landreth, G.E.; Sweatt, J.D. Mice lacking the ERK1 isoform of MAP kinase are unim-paired in emotional learning. Learn. Mem. 2001, doi:10.1101/lm.37001.
65. Shumyatsky, G.P.; Malleret, G.; Shin, R.M.; Takizawa, S.; Tully, K.; Tsvetkov, E.; Zakharenko, S.S.; Joseph, J.; Vronskaya, S.; Yin, D.Q.; et al. stathmin, a gene enriched in the amygdala, controls both learned and innate fear. Cell 2005, doi:10.1016/j.cell.2005.08.038.
66. Wehner, J.M.; Radcliffe, R.A. Cued and contextual fear conditioning in mice. Curr. Protoc. Neurosci. 2004, doi:10.1002/0471142301.ns0805cs27.
67. Novick, A.M.; Mears, M.; Forster, G.L.; Lei, Y.; Tejani-Butt, S.M.; Watt, M.J. Adolescent social defeat alters N-methyl-d-aspartic acid receptor expression and impairs fear learning in adulthood. Behav. Brain Res. 2016, 304, 51–59, doi:10.1016/j.bbr.2016.02.013.
68. MacKay, J.C.; Kent, P.; James, J.S.; Cayer, C.; Merali, Z. Ability of palatable food consumption to buffer against the short- and long-term behavioral consequences of social defeat exposure during juvenility in rats. Physiol. Behav. 2017, doi:10.1016/j.physbeh.2017.04.002.
69. Patki, G.; Solanki, N.; Atrooz, F.; Ansari, A.; Allam, F.; Jannise, B.; Maturi, J.; Salim, S. Novel mechanistic insights into treadmill exercise based rescue of social defeat-induced anxiety-like behavior and memory impairment in rats. Physiol. Be-hav. 2014, doi:10.1016/j.physbeh.2014.04.011.
70. Patki, G.; Solanki, N.; Atrooz, F.; Allam, F.; Salim, S. Depression, anxiety-like behavior and memory impairment are associ-ated with increased oxidative stress and inflammation in a rat model of social stress. Brain Res. 2013, doi:10.1016/j.brainres.2013.09.033.
71. Riga, D.; Theijs, J.T.; De Vries, T.J.; Smit, A.B.; Spijker, S. Social defeat-induced anhedonia: Effects on operant sucrose-seeking behavior. Front. Behav. Neurosci. 2015, doi:10.3389/fnbeh.2015.00195.
72. Verbitsky , A.; Dopfel, D.; Zhang, N. Rodent models of post-traumatic stress disorder: Behavioral assess-ment. Transl. Psychiatry 2020, 10, 1–28.
73. Lisieski , M.J.; Eagle, A.L.; Conti, A.C.; Liberzon, I.; Perrine, S.A. Single-prolonged

stress: A review of two decades of progress in a rodent model of post-traumatic stress disorder. Front. Psychiatry 2018, 9, 196.
74. Souza , R.R.; Noble, L.J.; McIntyre, C.K. Using the single prolonged stress model to examine the patho-physiology of PTSD. Front. Pharmacol. 2017, 8, doi:10.3389/fphar.2017.00615.
75. Iwamoto, Y.; Morinobu, S.; Takahashi, T.; Yamawaki, S. Single prolonged stress increases contextual freezing and the ex-pression of glycine transporter 1 and vesicle-associated membrane protein 2 mRNA in the hippocampus of rats. Prog. Neuro Psychopharmacol. Biol. Psychiatry 2007, 31, 642–651, doi:10.1016/j.pnpbp.2006.12.010.
76. Knox, D.; George, S.A.; Fitzpatrick, C.J.; Rabinak, C.A.; Maren, S.; Liberzon, I. Single prolonged stress disrupts retention of extinguished fear in rats. Learn. Mem. 2012, 19, 43–49, doi:10.1101/lm.024356.111.
77. Wu, Z.; Tian, Q.; Li, F.; Gao, J.; Liu, Y.; Mao, M.; Liu, J.; Wang, S.; Li, G.; Ge, D.; et al. Behavioral changes over time in post-traumatic stress disorder: Insights from a rat model of single prolonged stress. Behav. Processes 2016, 124, 123–129, doi:10.1016/j.beproc.2016.01.001.
78. Jovanovic, T.; Blanding, N.Q.; Norrholm, S.D.; Duncan, E.; Bradley, B.; Ressler, K.J. Childhood abuse is associated with in-creased startle reactivity in adulthood. Depress. Anxiety 2009, 26, 1018–1026.
79. Jovanovic, T.; Norrholm, S.D.; Fennell, J.E.; Keyes, M.; Fiallos, A.M.; Myers, K.M.; Davis, M.; Duncan, E.J. Posttraumatic stress disorder may be associated with impaired fear inhibition: Relation to symptom severity. Psychiatry Res. 2009, doi:10.1016/j.psychres.2007.12.014.
80. Bryant, R.A. Post-traumatic stress disorder: A state-of-the-art review of evidence and challenges. World Psychiatry 2019, doi:10.1002/wps.20656.
81. Vollmayr, B.; Faust, H.; Lewicka, S.; Henn, F.A. Brain-derived-neurotrophic-factor (BDNF) stress response in rats bred for learned helplessness. Mol. Psychiatry 2001, doi:10.1038/sj.mp.4000907.
82. Coppens, C.M.; Sipornmongcolchai, T.; Wibrand, K.; Alme, M.N.; Buwalda, B.; de Boer, S.F.; Koolhaas, J.M.; Bramham, C.R. Social defeat during adolescence and adulthood differentially induce BDNF-regulated immediate early genes. Front. Behav. Neurosci. 2011, doi:10.3389/fnbeh.2011.00072.
83. Toth, E.; Gersner, R.; Wilf-Yarkoni, A.; Raizel, H.; Dar, D.E.; Richter-Levin, G.; Levit, O.; Zangen, A. Age-dependent effects of chronic stress on brain plasticity and depressive behavior. J. Neurochem. 2008, 107, 522–532, doi:10.1111/j.1471-4159.2008.05642.x.
84. Montaron, M.F.; Piazza, P.V.; Aurousseau, C.; Urani, A.; Le Moal, M.; Abrous, D.N. Implication of corticosteroid receptors in the regulation of hippocampal structural plasticity. Eur. J. Neurosci. 2003, doi:10.1111/j.1460-9568.2003.03048.x.
85. Nacher, J.; Lanuza, E.; McEwen, B.S. Distribution of PSA-NCAM expression in the amygdala of the adult rat. Neuroscience 2002, doi:10.1016/S0306-4522(02)00219-1.
86. Tzanoulinou, S.; Gantelet, E.; Sandi, C.; Márquez, C. Programming effects of peripubertal

stress on spatial learning. Neurobi-ol. Stress 2020, doi:10.1016/j.ynstr.2020.100282.
87. Tsoory, M.; Guterman, A.; Richter-Levin, G. Exposure to stressors during juvenility disrupts development-related altera-tions in the PSA-NCAM to NCAM expression ratio: Potential relevance for mood and anxiety disorders. Neuropsychophar-macology 2008, doi:10.1038/sj.npp.1301397.
88. Leal, G.; Afonso, P.M.; Salazar, I.L.; Duarte, C.B. Regulation of hippocampal synaptic plasticity by BDNF. Brain Res. 2015, 1621, 82–101.
89. Cohen-Cory, S.; Kidane, A.H.; Shirkey, N.J.; Marshak, S. Brain-derived neurotrophic factor and the development of struc-tural neuronal connectivity. Dev. Neurobiol. 2010, 70, 271–288.
90. Huang, E.J.; Reichardt, L.F. Neurotrophins: Roles in neuronal development and function. Annu. Rev. Neurosci. 2001, 24, 677–736.
91. Wu, Y.; Liu, Q.; Guo, B.; Ye, F.; Ge, J.; Xue, L. BDNF activates postsynaptic TrkB receptors to induce endocannabinoid release and inhibit presynaptic calcium influx at a calyx-type synapse. J. Neurosci. 2020, doi:10.1523/JNEUROSCI.2838-19.2020.
92. Daskalakis, N.P.; Kloet, E.R. De; Yehuda, R.; Malaspina, D.; Kranz, T.M. Early life stress effects on glucocorticoid—BDNF in-terplay in the hippocampus. Front. Mol. Neurosci. 2015, 8, 1–13.
93. Santa Ana, E.J.; Saladin, M.E.; Back, S.E.; Waldrop, A.E.; Spratt, E.G.; McRae, A.L.; LaRowe, S.D.; Timmerman, M.A.; Upadh-yaya, H.; Brady, K.T. PTSD and the HPA axis: Differences in response to the cold pressor task among individuals with child vs. adult trauma. Psychoneuroendocrinology 2006, doi:10.1016/j.psyneuen.2005.11.009.
94. Suzuki, A.; Poon, L.; Papadopoulos, A.S.; Kumari, V.; Cleare, A.J. Long term effects of childhood trauma on cortisol stress reactivity in adulthood and relationship to the occurrence of depression. Psychoneuroendocrinology 2014, doi:10.1016/j.psyneuen.2014.09.007.

CHAPTER 6

LONG-TERM SEX-DEPENDENT EFFECTS OF REPEATED BRIEF SOCIAL ISOLATION STRESS DURING EARLY ADOLESCENCE ON RESPONSE TO A SECOND CHALLENGE IN ADULT RATS

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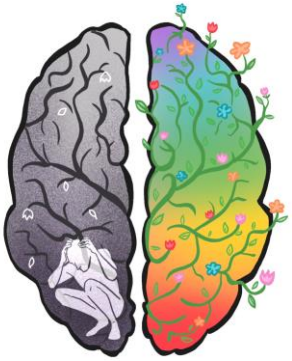
In preparation



Abstract

Social isolation stress (SIS) is a well-validated stress paradigm used to reproduce in rodents alterations resembling those of different psychiatric disorders. SIS is generally performed in male rats starting from adolescence until adulthood for long and continuative periods. However, the long-term effects of a briefer period of SIS occurring only during early adolescence and potential sex-differences in response to SIS are not investigated. Morevoer, early-life adverse experiences (first hit) may affect resilience or vulnerability to later experienced stressful events (second hit) and the subsequent development of stress-related psychopathologies. Male and female rats were subjected to daily 2 hours of SIS during early adolescence, and the emotional and cognitive effects were assessed in adulthood. Further, SIS-rats and their controls were additionally subjected to single prolonged stress (SPS) in adulthood and the behavioral outcomes were evaluated long after trama. We demonstrated that SIS induces hyperarousal and anxious-like phenotype only in male adult rats, and enhanced avoidance behavior in female adult rats. SPS induced a reduction of locomotor activity in both sexes, dysfunctions in spatial memory retention in males and altered fear memory dynamics in females. Rats experienced both stressors exhibited resilience towards alterations in emotionality induced by SIS exposure in males and towards SIS-induced avoidance behavior in females. The SPS altered locomotion and spatial memory retention; these effects were absent in SIS-exposed rats later exposed to SPS. Our findings show that briefer SIS influences the ability to cope with a second challenge experienced later in life in a sex-dependent manner.

Introduction

Adolescence represents the transition period between childhood and adulthood, during which individuals gain cognitive, emotional and social skills necessary to acquire autonomy and independence from parental caretakers (Spear, 2000). Social interactions and building a complex social tissue are the main characteristics of humans and most mammalians (Trezza et al., 2011). In particular, in humans the quality and quantity of social interactions are of outmost importance during adolescence (Larson et al., 1996). Evidence suggests that adolescent social deprivation can lead to the development of mental disorders later in life (Hankin et al., 1998; Orben et al., 2020; Patterson et al., 1992). Similar to humans, it is well established that the deprivation of social interactions with conspecifics profoundly affects behavior in adulthood in rodents (Blakemore and Mills, 2014; Fone and Porkess, 2008; Mumtaz et al., 2018; Trezza et al., 2010). In this scenario, animal models have been widely used to understand the neurobiological and behavioral effects induced by deprivation or reduction of social interactions. Particularly, social isolation stress (SIS) is the most commonly used stress paradigm to reproduce in rodents neurochemical and behavioral alterations resembling some of the core symptoms observed in schizophrenic patients (Fone and Porkess, 2008; Heidbreder et al., 2000). The SIS paradigm consists in housing animals individually with no handling, depriving them of any form of social interaction with peers. Traditionally, it is conducted chronically and continuously for long periods of time (e.g. 4-6 weeks or more) from weaning to adulthood (Heidbreder et al., 2000; Lapiz et al., 2003). Compelling evidence indicates that long periods of post-weaning SIS induce in male rats anxious-depressive-like phenotype and cognitive deficits (Butler et al., 2016; Han et al., 2018; Ieraci et al., 2016; Lukkes et al., 2009b; Medendorp et al., 2018; Mumtaz et al., 2018). However, these results are inconsistent between males and females rats (Walker et al., 2019). In fact, it appears that SIS exposure does not affect emotionality (Weintraub et al., 2010) while altering fear memory dynamics in female rats (Butler et al., 2016; Jahng et al., 2012; Weintraub et al., 2010; Weiss et al., 2004). Despite there are numerous preclinical studies based on long and continuative periods of SIS, it

remains difficult to reach a high translational value to humans by using this stress model. Nowadays understanding the effects of brief periods of SIS is more and more becoming an impelling issue. Indeed, spending daily brief and repeated periods on social platforms or playing video games has become a habit among the current generation of the adolescents and when it became chronic, it could represent a serious public health problem (Anderson et al., 2010; Müller et al., 2014; Von Der Heiden et al., 2019). However, social deprivation is not always voluntary. The physical distancing measures currently adopted to contain the spread of the COVID-19 forced many adolescents in the worldwide to avoid any form of face-to-face social contacts with peers, and it could probably result in the development of mental health problems in the log-term (Orben et al., 2020).

Recent literature data demonstrated that adolescents who daily spend hours playing videogames are on high risk to develop many psychological disturbances (Mihara and Higuchi, 2017). In fact, brief and repeated periods of SIS during adolescence could induce middle and subtle alterations that are not associated with a specific psychopathology and, consequentially, difficult to classify and diagnose. The effects induced by briefer period of SIS are underinvestigated. The only evidence available demonstrates that 6 hours of SIS per day from PND 21 to 29 increased immobility in the forced swim stress in adolescent male rats (Shetty and Sadananda, 2017), 6 hours of SIS per day from PND 15 to 21 enhanced spatial learning in adult rats (Frisone et al., 2002), and 1 hour of SIS per day from PND 33 to 48 did not affect sensitivity to drugs of abuse in male but not female adult rats (McCormick et al., 2005). Further, 1 hour of SIS per day from PND 30 to 45 induced memory alterations paralleled by a reduced hippocampal cell proliferation in female in adulthood (McCormick et al., 2010). Thus, compared to the large body of literature analyzing the effects of long and continuative periods of SIS in rodents, studies evaluating the long-term effects induced by repeated brief periods of SIS during early adolescence, which is a critical window for brain development, and whether these effects are sex-dependent are lacking.

Exposure to adverse stimuli is one of the most important environmental factor critically

involved in the individual difference in the response of stress (Andolina et al., 2015). Individuals can be classified into two categories on the basis of their reaction to stress: vulnerable people that negatively respond to adverse stressful stimuli, and resilient people that positively respond to the same stimuli (Daskalakis et al., 2015; McEwen, 2016).Various hypotheses have been postulated to explain this difference between individuals. The "two-hit" stress model has been used to investigate whether exposure to two different stressors at different ages increases or decreases the risk of developing psychopathologies after experiencing the second stressor (Faraji et al., 2017; Hill et al., 2014; Peña et al., 2017; Tsoory et al., 2007). The effects of brief and repeated periods of SIS in humans experienced during adolescence on the reaction to an additional stressor later in life, however, has not yet been investigated. To elucidate this issue, we exposed male and female rats to 2 hours of SIS during early adolescence (PND 28-34) and then exposed them in adulthood to a single episode of prolonged stress (SPS) as a second stressor. SPS is a validated stress paradigm comprising 3 stressful events presented during a single session (Kohda et al., 2007; Liberzon et al., 1999, 1997). Here, we evaluated whether exposure to stress during early adolescence (first hit) affects emotionality and cognitive processes in the long-term and whether exposure to a second challenge (second hit) later in life alters such effects. Additionally, we investigated whether these behavioral alterations are sex dependent.

Therefore, male and female rats were exposed to repeated brief SIS during early adolescence and to SPS in adulthood, and then subjected to a behavioral test battery, including the open field, elevated plus maze, acoustic startle response, marble burying, Morris water maze, and auditory fear conditioning.

Materials and Methods

Animal care and use

Male and female Sprague-Dawley rats at PND 21 (Charles River Laboratories, Calco, Italy) were housed in air-conditioned vivarium rooms (temperature: 21 ± 1°C; lights on

from 7:00 am to 7:00 pm) with pellet food and water available ad libitum. Rats were housed in groups of 4 per cage until postnatal day (PND) 70 when animals of the same treatment and sex were pair-housed. All the experiments were performed during the light phase of the cycle. All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, and the D. L. 26/2014 of Italian Ministry of Health.

Social isolation stress protocol

Separate groups of male and female rats were randomly assigned into either SIS (SIS-CTRL) or control (CTRL-CTRL) groups. Rats assigned to SIS-CTRL group were removed from their home cage and singularly housed in a novel one for 2 hours per day for 7 consecutive days from PND 28 to 34. After SIS procedure, SIS-CTRL rats were returned in the home cage with their conspecifics. The correspondent CTRL-CTRL rats were left undisturbed in their home cage during SIS protocol in a separate room.

Single prolonged stress protocol

We used a previously described SPS protocol (Mancini et al., 2020; Ganon-Elazar and Akirav, 2012a; Liberzon et al., 1997) with slight modification. Briefly, male and female rats at PND 90 were exposed to a single session of SPS that consisted of 2 h of restraint stress in a restrainer (30 cm in length with an inside diameter of 7.5 cm), 15 min of forced swim stress (24±1 °C water temperature) followed by 15 min of recovery, and then isoflurane exposure until loss of consciousness. Rats not assigned to SPS groups were placed in a separate room throughout the duration of SPS. After the SPS procedure, all rats were returned to their home cage and left undisturbed for 30 days.

Experimental design

As shown in Fig. 1, adolescent rats at PND 28 were randomly assigned to one of 4 groups:

- CTRL-CTRL group: rats not exposed to any stressors

- SIS-CTRL group: rats exposed to only SIS during the early adolescence (PND 28-34)
- CTRL-SPS group: rats exposed to only SPS at PND 90
- SIS-SPS group: rats exposed to SIS during adolescence and SPS at PND 90

Animals were left undisturbed for 30 days after SPS exposure and then exposed to a behavioral test battery, including open field, acoustic startle response, elevated plus maze, marble burying, Morris water maze and auditory fear conditioning tasks (see Fig. 1).

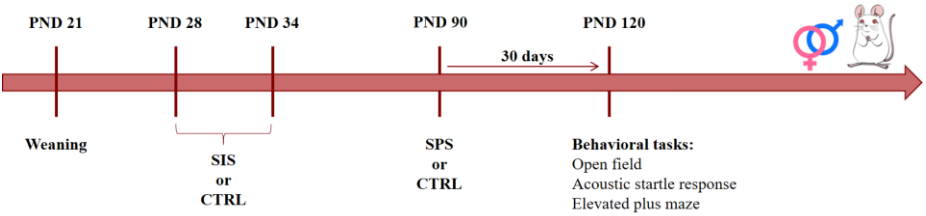


Figure 1: Experimental timeline. Separate cohorts of male and female rats at PND 28 were randomly assigned to either the CTRL-CTRL, SIS-CTRL, CTRL-SPS, or SIS-SPS groups. Rats were subjected to 2 hours of SIS daily for 7 consecutive days from PND 28 to 34, and then to the SPS paradigm in adulthood (PND 90). Thirty days after SPS exposure, separate groups of rats were exposed to a behavioral test battery, including open field, acoustic startle response, elevated plus maze, marble burying, Morris water maze and auditory fear conditioning tasks.

Behavioral tasks

Open field

In the open field test each rat was placed into the corner of the open field arena (80 × 80 × 60 cm). The test was performed under dim light conditions (2 lux) and lasted 20 min, as previously described (Colucci et al., 2020). The total distance traveled (cm) by each rat was determined as an index of the rat locomotor activity. After each session, rats were returned to their home cage, fecal boli were removed and the walls and floor of the arena were cleaned with a 70% ethanol solution. The total distance traveled was acquired and analyzed using an automated video-tracking system (Smart, Panlab,

Barcelona, Spain).

Acoustic startle response

A slightly modified procedure as the one previously described (Ganon-Elazar and Akirav, 2012b) was used. Rats were placed in a startle reflex apparatus (Med Associates, Fairfax, Vermont) for a 5 min acclimatization period with a 70 dB background noise, which continued during all the session. Each session consisted of 10 pulse trials (115 dB) with intertrial intervals selected randomly between 10 and 15 s. Acoustic devices and startle cages were connected to a computer, which detected and analyzed all chamber variables using customized software (Med Associates, Fairfax, Vermont). The system allows recording and analysis of the signal generated by the animal movement through a high sensitivity weight transducer system. The maximal startle reflex response for each animal was calculated as the average of the responses to the 10 pulse trials.

Elevated plus maze

The test was performed as previously described (Almenrader et al., 2017; Morena et al., 2016). The elevated plus maze apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) that extended from a common central platform (10 × 10 cm). The apparatus, made of plexiglass (black floor and walls), was elevated to a height of 60 cm above the floor level. Rat's behavior was recorded for 5 min by using a video camera positioned above the experimental apparatus and videos were analyzed with Observer XT 12 (Noldus Information Technology BV, Wageningen, The Netherlands) by observers blinded to the experimental conditions. Each rat was individually placed on the central platform facing a closed arm. Time spent in the open arms (s) and number of entries in the open arms were analyzed. After each session, rats were returned to their home cage, fecal boli were removed and the apparatus was cleaned with a 70% ethanol solution.

Marble burying

We used a slightly modified protocol as the one previously described (Zanda et al., 2017). The marble burying test was conducted in a quadrangular arena (40 × 40 × 60 cm) made of plexiglass with clean sawdust covering the floor, under dim light condition. Twenty-five standard glass marbles (1.5 cm diameter, arranged in five rows of five marbles each) were placed uniformly over the surface. Individual rats were placed in the marble arena and activity was monitored for 30 min by a video camera placed above the arena. At the end of the session, animals were gently removed from the arena and returned to their home cage, and the number of marbles buried was counted. New bedding was used for each animal, and marbles were cleaned with 70% ethanol solution.

Morris water maze

The experimental apparatus was a circular tank (1.83 m in diameter and 0.58 m in height) filled with water (23–24 °C) to a depth of 20 cm. The maze was located in a room containing many salient, visual, extra-maze cues. During the spatial training a rectangular platform (20 x 25 cm) was placed at a fixed location 25 cm away from the edge of the pool and 2.5 cm below the water surface. The experiments were performed according to the procedure previously described (Morena et al., 2015; Scuderi et al., 2014). For spatial training, the rats were given four trials on each daily session for three consecutive days. On each trial, the animal was placed in the tank facing the wall at one of the 4 designated start positions and allowed to escape onto the hidden platform. If an animal failed to find the platform within 60 s during the first day of the training, it was manually guided to the platform. The rat was allowed to remain on the platform for 10 s and was then placed into a holding cage for 25 s until the start of the next trial. The time each animal spent to reach the platform was recorded as the escape latency. Retention of the spatial training was assessed 24 h after the last training session with a 60 s free-swim probe trial and a new starting position was used. Training and probe trials were videotaped, and an automated tracking system (Smart, Panlab, Barcelona,

Spain) was used to analyze the swim path of each subject and calculate the initial latency to cross the platform location and the number of crossings through the platform location. The target and opposite quadrants were equidistant from the starting position on the probe trial.

Auditory fear conditioning

We used a slightly modified protocol as previously described (Atsak et al., 2012; Rodriguez-Romaguera et al., 2009). All the phases of the auditory fear conditioning task were performed in the same operant chambers, located in sound-attenuating cubicles (Med Associates, Fairfax, Vermont). The floor of the chambers consisted of stainless-steel bars that delivered a scrambled electric footshock. On day 1, rats received 5 habituation tones (30 s, 4 kHz, 75 dB; 3 min intertrial interval), immediately followed by 7 conditioning tones that co-terminated with footshocks (0.5 s, 0.65 mA). On day 2, rats were returned to the chambers for the extinction session, which consisted of 12 tones in the absence of footshock. On day 3, rats were returned to the chambers and presented with 8 tones in the absence of footshock to test for extinction retrieval. Freezing behavior, defined as the absence of all movements except for those related to breathing (Blanchard and Blanchard, 1972), served as the measurement of fear and was monitored with digital video cameras. An experimenter blinded to the experimental conditions quantified the total time that rats spent freezing during the 30 s tone presentations by using a digital stopwatch. The percentage of time spent in freezing during the 30 s tone presentations was calculated as: [time spent in freezing during the tone presentation (s) / 30 s] x 100. For the conditioning session (day 1), the time spent in freezing during the first and the last tone presentation was represented, while for day 2 and 3 the average of percentage of time spent in freezing during all trials was represented.

Statistical analysis

Statistical analysis was performed using SPSS statistical software. Data are expressed

as mean ± SEM. Escape latencies during the acquisition phase of the Morris water maze and the percentage of freezing during the conditioning phase of the auditory fear conditioning were analyzed with a repeated measures ANOVA (RM ANOVA). In all the other cases, data were analyzed with two-way ANOVA. Tukey-Kramer post-hoc tests were performed to control for significant differences between groups when appropriate. A probability level of < 0.05 was accepted as statistically significant.

Results

The interaction between SIS during early adolescence and SPS at adulthood induces resilience towards hyperarousal and anxious behavior later in life in males and resilience towards avoidance behavior in females

Open field test

In males, as shown in Fig. 2a, two-way ANOVA for the distance traveled in the open field showed no significant effect of SIS ($F_{(1,35)} = 2.223$; $P = 0.145$), but significant effects of SPS ($F_{(1,35)} = 5.817$; $P = 0.021$) and SIS x SPS interaction ($F_{(1,35)} = 4.617$; $P = 0.039$). Post hoc analysis revealed that rats exposed to SPS only traveled shorter distance compared with control rats and rats exposed to both stressors ($P < 0.05$). Two-way ANOVA for the time spent in the center of the open field arena showed no significant effect of SIS ($F_{(1,35)} = 1.526$; $P = 0.225$), but showed significant effects of SPS ($F_{(1,35)} = 4.879$; $P = 0.034$) and SIS x SPS interaction ($F_{(1,35)} = 6.730$; $P = 0.014$; Fig. 2b). Post hoc analysis revealed that rats exposed only to SIS spent less time in the center of the open field arena with respect to control rats ($P < 0.05$) and to rats exposed to both stressors ($P < 0.05$). Post hoc analysis revealed that rats exposed to both stressors spent more time in the center of the open field arena with respect to rats exposed to both SIS or SPS groups ($P < 0.05$).

In female rats, as shown in Fig. 2d, two-way ANOVA for the distance traveled in the open field showed no significant effect of SIS ($F_{(1,40)} = 0.011$; $P = 0.915$), a significant

effect of SPS ($F_{(1,40)}$ = 19.907; P < 0.0001) but no significant effect of SIS x SPS interaction ($F_{(1,40)}$ = 1.823; P = 0.185). Post hoc analysis indicated that female rats exposed to SPS present shorter distance traveled compared to the correspondent group of control (P < 0.05). Two-way ANOVA for the time spent in the center of the open field arena showed no significant effect of SIS ($F_{(1,40)}$ = 0.589; P = 0.447), a significant effect of SPS ($F_{(1,40)}$ = 13.439; P = 0.001) but no significant effect of SIS x SPS interaction ($F_{(1,40)}$ = 0.553; P = 0.462; Fig. 2e).

These results indicate that exposure to repeated brif periods of SIS during early adolescence did not alter locomotor activity in both male and female adult rats, while experiencing SPS in adulthood reduced locomotion in both sexes. However, exposure to both stressors induced resilience towards altered locomotor activity induced by SPS in males but not in females. Moreover, increased anxiety-like profile was found only in male adult rats exposed to SIS during early adolescence and that the additional exposure to SPS reverted such effects.

3.1.2 Acoustic startle response

In male rats, as shown in Fig. 2c, two-way ANOVA for the mean startle amplitude showed no significant effect of SIS ($F_{(1,35)}$ = 0.662; P = 0.421), or SPS ($F_{(1,35)}$ = 0.054; P = 0.817), but a significant effect of SIS x SPS interaction ($F_{(1,35)}$ = 9.382; P = 0.004). Post hoc analysis revealed that rats singularly exposed to SIS or to SPS reported higher mean startle amplitude than control rats (P < 0.01 and P < 0.05, respectively). Conversely, rats subjected to both stressors showed lower mean startle amplitude than rats only exposed to SPS (P < 0.05) and similar to that of control rats never exposed to any stressor.

In female rats, as shown in Fig. 2f, two-way ANOVA for the mean startle amplitude showed no significant effects of SIS ($F_{(1,42)}$ = 0.042; P = 0.840), SPS ($F_{(1,42)}$ = 2.434; P = 0.126), and SIS x SPS interaction ($F_{(1,42)}$ = 0.017; P = 0.897).

These results indicate that rats singularly exposed to SIS during early adolescence or to SPS in adulthood show hyperarousal and increased anxious-like profile later in life and

that such effects are absent when rats are subjected to both stressors, thus demonstrating that SIS during early adolescence together with the second challenge experienced later in life promotes resilience towards the development of emotional alterations. Conversely, female rats exposed to adolescent SIS or adult SPS did not show hyperarousal and anxiety-like behavior later in life.

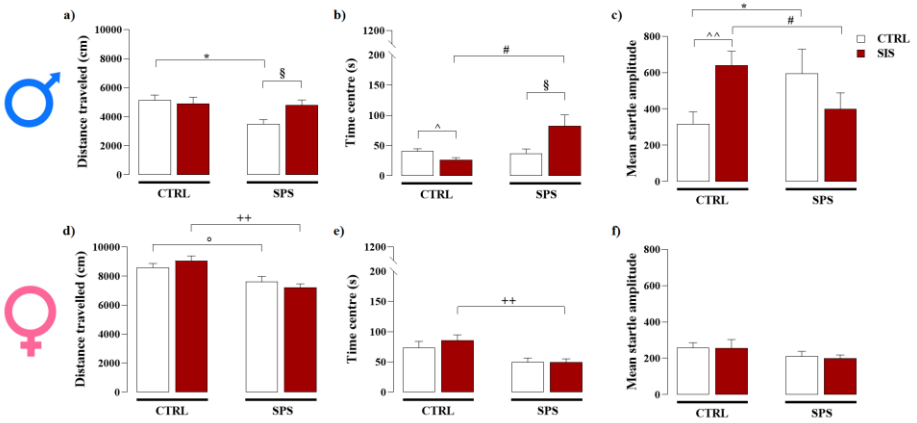


Figure 2: Long-term and sex-dependent effects on locomotor activity, anxiety-like behavior and hyperarousal induced by SIS, SPS, or the combination of both stressors. Long-term effects induced by SIS, SPS, or both stressors in males on the a) distance traveled (cm) evaluated in the open field arena, b) time spent in the center of the arena in the open field, and c) mean startle amplitude in the acoustic startle response task. *, $P < 0.05$ vs. CTRL-CTRL group; §, $P < 0.05$ vs. CTRL-SPS group; #, $P < 0.05$ vs. SIS-CTRL group; ^, $P < 0.05$ vs. CTRL-CTRL group; ^^, $P < 0.01$ vs. CTRL-CTRL group. (N = 10-11 rats per group or N = 8-12 rats per group, in the open field or acoustic startle response tasks, respectively). Long-term effects induced by SIS, SPS, or both stressors in females on the d) distance traveled (cm) evaluated in the open field arena, e) time spent in the center of the arena in the open field, and f) mean startle amplitude in the acoustic startle response task. °, $P < 0.05$ vs. CTRL-CTRL group; ++, $P < 0.01$ vs. SIS-CTRL group. (N = 11 rats per group or N = 11-12 rats per group, in the open field or acoustic startle response tasks, respectively).

Elevated plus maze

As shown in Fig. 3a, two-way ANOVA for the time spent in the open arms by male rats showed no significant effect of SIS ($F_{(1,35)} = 0.171$; $P = 0.681$), SPS ($F_{(1,35)} = 3.398$; $P = 0.074$) but showed a significant effect of SIS x SPS interaction ($F_{(1,35)} = 9.369$; $P = 0.004$). Post hoc analysis revealed that rats singularly exposed to either SIS or SPS spent less time in the open arms with respect to control rats ($P < 0.05$ and $P < 0.01$, respectively). Post hoc analysis revealed that male rats exposed to both stressors spent more time in the open arms with respect to rats exposed to SPS alone ($P < 0.05$) and to control rats never exposed to any stressor. Two-way ANOVA for the number of entries in the open arms showed no significant effect of SIS ($F_{(1,35)} = 0.012$; $P = 0.915$), a significant effects of SPS ($F_{(1,35)} = 4.228$; $P = 0.047$) and a significant SIS x SPS interaction ($F_{(1,35)} = 5.075$; $P = 0.031$; Fig. 3b). Post hoc analysis indicated that rats singularly exposed to SPS presented less open arm entries compared to CTRL-male rats ($P < 0.05$). On the contrary, rats exposed to both stressors reported a number of open arms entries similar to that of CTRL-male rats.

Aa shown in Fig. 3d, two-way ANOVA for the time spent in the open arms by female rats showed a significant effect of SIS ($F_{(1,40)} = 5.078$; $P = 0.030$), but no significant effects of SPS ($F_{(1,40)} = 1.977$; $P = 0.167$) and SIS x SPS interaction ($F_{(1,40)} = 0.078$; $P = 0.782$). Two-way ANOVA for the number of entries in the open arms showed no significant effect of SIS ($F_{(1,40)} = 2.663$; $P = 0.111$), SPS ($F_{(1,40)} = 0.426$; $P = 0.518$) and SIS x SPS interaction ($F_{(1,40)} = 0.027$; $P = 0.871$; Fig. 3e).

These results indicate that SIS experienced during early adolescence or SPS in adulthood induced an anxious-like profile in adult male rats and that these effects were reverted when rats were exposed to both stressors. Conversely, female rats exposed to adolescent SIS or SPS at adulthood did not show any alteration on anxiety-like behavior in the long-term.

Marble burying

As shown in Fig. 3c, two-way ANOVA for the number of marbles buried by male rats

showed no significant effects of SIS ($F_{(1,35)} < 0.001$; P = 0.996), SPS ($F_{(1,35)} = 2.380$; P = 0.132) and SIS x SPS interaction ($F_{(1,35)} = 2.775$; P = 0.105).

Two-way ANOVA for the number of marbles buried by female rats showed no significant effects of SIS ($F_{(1,41)} = 1.453$; P = 0.235), SPS ($F_{(1,41)} = 1.815$; P = 0.185), but a significant effect of SIS x SPS interaction ($F_{(1,41)} = 6.159$; P = 0.017; Fig. 3f). Post hoc analysis revealed that the number of marbles buried by SIS-female rats was higher than in CTRL-female rats (P < 0.05). Post hoc analysis revealed that the number of marbles buried by rats exposed to both stressors was less than in SPS-female rats (P < 0.05) and similar to that of control rats never exposed to any stressor.

These results indicate that SIS during early adolescence increased avoidance behavior in female but not male rats in the long-term. Conversely, rats exposed to SPS exhibited avoidance behavior in male but not female rats. Furthermore, exposure to both stressors reduced the number of marbles buried in both sexes, indicating resilience towards avoidance and obsessive-compulsive like behavior later in life.

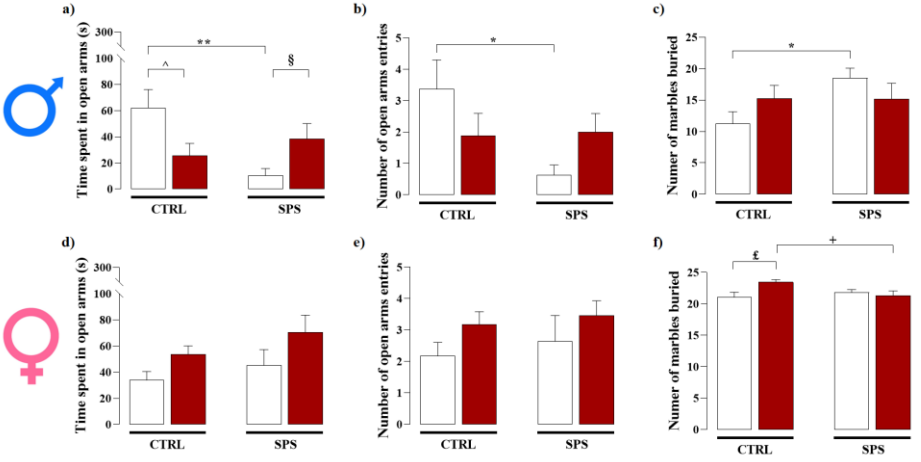


Figure 3: Long-term and sex-dependent effects on anxiety-like behavior and avoidance induced by SIS, SPS, or the combination of both stressors. Long-term effects induced by SIS, SPS, or both stressors in males on the a) time spent in the open arms in the elevated plus maze, b) number of open arms entries in the elevated plus maze, and c) number of marbles buried in the

marble burying task. *, $P < 0.05$ vs. CTRL-CTRL group; **, $P < 0.01$ vs. CTRL-CTRL group; §, $P < 0.05$ vs. CTRL-SPS group; ^, $P < 0.05$ vs. CTRL-CTRL group. (N = 8-12 rats per group or N = 8-12 rats per group, in the elevated plus maze or marble burying tasks, respectively). Long-term effects induced by SIS, SPS, or both stressors in females on the d) time spent in the open arms in the elevated plus maze, e) number of open arms entries in the elevated plus maze, and f) number of marbles buried in the marble burying task. £, $P < 0.05$ vs. CTRL-CTRL group; +, $P < 0.05$ vs. SIS-CTRL group. (N = 11 rats per group or N = 11-12 rats per group, in the elevated plus maze or marble burying tasks, respectively).

The interaction between SIS during early adolescence and SPS at adulthood induces resilience towards spatial memory deficits later in life in males

As shown in Fig. 4a, RM ANOVA for escape latency during spatial training of male rats revealed a significant effect of trials ($F_{(2,72)} = 148.131$; $P < 0.0001$), indicating that all groups progressively learned to locate the platform across the 3 training days (no other statistical differences were found). Two-way ANOVA for the initial latency to cross the platform location during the probe trial indicated significant effects of SIS ($F_{(1,36)} = 5.273$; $P = 0.028$), SPS ($F_{(1,36)} = 5.388$; $P = 0.026$) and interaction between these two factors ($F_{(1,36)} = 13.577$; $P = 0.001$; Fig. 4b). Post hoc analysis revealed that the latency to cross the platform location of rats only exposed to SPS was higher than that of the control group ($P < 0.01$) and those of rats subjected to both stressors ($P < 0.01$). Two-way ANOVA for the number of crossings through the platform location revealed no significant effect of SIS ($F_{(1,36)} = 0.080$; $P = 0.779$), a significant effect of SPS ($F_{(1,36)} = 8.358$; $P = 0.007$) but a not significant SIS x SPS interaction ($F_{(1,36)} = 2.268$; $P = 0.141$; Fig. 4c). Post hoc analysis showed that the number of crossings through the platform location of rats only exposed to SPS was lower than that of the control group ($P < 0.05$).

RM ANOVA for escape latency during spatial training of female rats revealed a significant effect of trials ($F_{(2,80)} = 98.862$; $P < 0.0001$; Fig. 4d), indicating that all groups progressively learned to locate the platform across the 3 training days (no other

statistical differences were found). For retention memory during the probe trial two-way ANOVA for the initial latency to cross the platform location indicated no significant effect of SIS ($F_{(1,40)}$ = 0.026; P = 0.874), SPS ($F_{(1,40)}$ = 0.378; P = 0.542), and the interaction between these two factors ($F_{(1,40)}$ = 0.038; P = 0.847; Fig. 4e). Two-way ANOVA for the number of crossings through the platform location revealed no significant effect of SIS ($F_{(1,40)}$ = 0.977; P = 0.329), SPS ($F_{(1,40)}$ = 0.997; P = 0.329) and the interaction between these two factors ($F_{(1,40)}$ = 0.039; P = 0.844; Fig. 4f).

These results demonstrate that SIS during early adolescence did not affect spatial memory functions in both sexes in the long-term. Additionally, male but not female rats singularly exposed to SPS had spatial memory retention deficits. Further, male rats that experienced both SIS during early adolescence and SPS at adulthood did not show cognitive deficits.

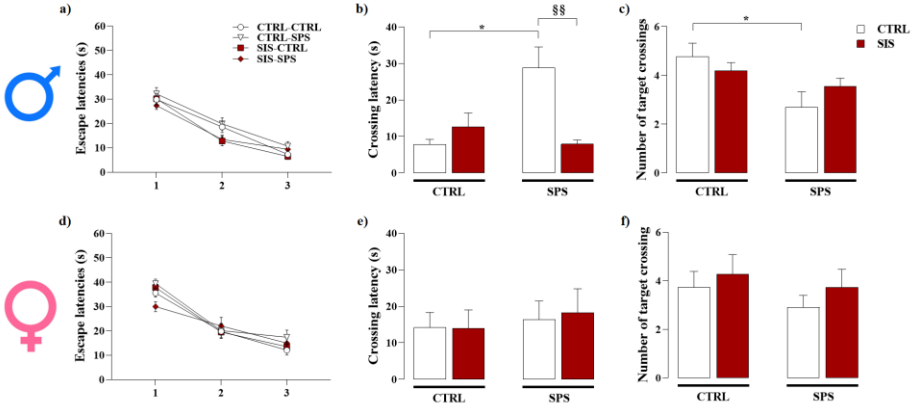


Figure 4: Long-term effects on spatial memory induced by SIS, SPS, or the combination of both stressors. Long-term effects induced by SIS, SPS, or both stressors in males in the Morris water maze task on a) escape latencies across trials during each day of the acquisition phase, b) the latency to cross the platform location, and c) the number of crossings over the platform location during the probe session. *, P < 0.05 vs. CTRL-CTRL group; §§, P < 0.01 vs. CTRL-SPS group. (N = 9-11 rats per group). Long-term effects induced by SIS, SPS, or both stressors in females in the Morris water maze task on d) escape latencies across trials during each day of

the acquisition phase, e) the latency to cross the platform location, and f) the number of crossings over the platform location during the probe session. (N = 11 rats per group).

The interaction between SIS during early adolescence and SPS at adulthood induces resilience towards cued fear memory deficits later in life in females

As shown in Fig. 5a, RM ANOVA for the percentage of freezing of male rats during fear conditioning acquisition (day 1) revealed a significant main effect of tone presentation ($F_{(1,38)} = 21.285$; $P < 0.0001$), indicating that all groups learned to associate the tone with the footshock (no other statistical differences were found). Two-way ANOVA for the time spent in freezing during the extinction phase on day 2 (Fig. 5b) revealed no significant effects of SIS ($F_{(1,38)} = 0.249$; $P = 0.621$), SPS ($F_{(1,38)} = 0.841$; $P = 0.365$), and SIS x SPS interaction ($F_{(1,38)} = 0.632$; $P = 0.432$). Two-way ANOVA for the time spent in freezing during the memory retrieval session on day 3 (Fig. 5c), showed no significant effects of SIS ($F_{(1,38)} = 0.007$; $P = 0.934$), SPS ($F_{(1,38)} = 1.356$; $P = 0.252$), and SIS x SPS interaction ($F_{(1,38)} = 1.203$; $P = 0.280$).

As shown in Fig. 5d, RM ANOVA for the percentage of freezing of female rats during fear conditioning acquisition (day 1) did not reveal any significant effects of SIS ($F_{(1,28)} = 0.025$; $P = 0.875$), SPS ($F_{(1,28)} = 0.993$; $P = 0.328$) or interaction between these two factors ($F_{(1,28)} = 2.128$; $P = 0.156$), indicating that females did not learn to associate the tone with the footshock. Two-way ANOVA for the time spent in freezing during the extinction phase on day 2 (Fig. 5e) revealed no significant effects of SIS ($F_{(1,28)} = 0.281$; $P = 0.600$), SPS ($F_{(1,28)} = 0.005$; $P = 0.943$), and SIS x SPS interaction ($F_{(1,28)} = 0.097$; $P = 0.758$). Two-way ANOVA for the time spent in freezing during the memory retrieval session on day 3 (Fig. 5f), showed no significant main effects of SIS ($F_{(1,28)} = 0.333$; $P = 0.568$), SPS ($F_{(1,28)} = 4.856$; $P = 0.036$), and SIS x SPS interaction ($F_{(1,28)} = 5.910$; $P = 0.022$). Post hoc analysis showed a lower percentage of freezing in female rats exposed to SPS as compared to control and to females exposed to both stressors ($P < 0.05$).

These results indicate that exposure to SIS during early adolescence did not alter cued

fear memory dynamics in both male and female adult rats, and that SPS affected extinction retrieval only in female adult rats. Further, when female rats were exposed to both stressors fear memory dysfunctions induced by SPS were not detected, thus indicating that SIS during early adolescence together with the second challenge experienced later in life promotes resilience towards altered fear memory dynamics.

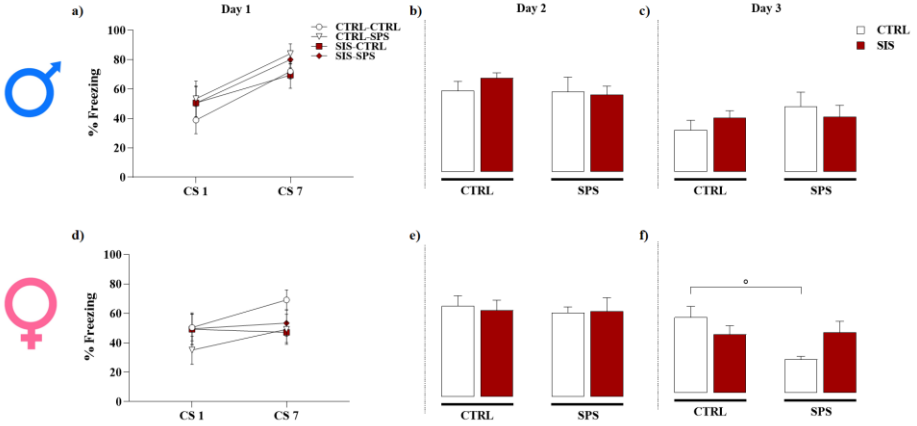


Figure 5: Long-term effects on cued fear memory dynamics induced by SIS, SPS, or the combination of both stressors. Long-term effects induced by SIS, SPS, or both stressors in males in the auditory fear conditioning paradigm on a) percentage of time spent in freezing during the first and last tone presentations at conditioning (day 1), b) mean percentage of time spent freezing during all 12 trials at extinction (day 2), c) mean percentage of time spent freezing during all 8 trials at extinction retrieval (day 3). (N = 9-12 rats per group). Long-term effects induced by SIS, SPS, or both stressors in females in the auditory fear conditioning paradigm on d) percentage of time spent in freezing during the first and last tone presentations at conditioning (day 1), e) mean percentage of time spent freezing during all 12 trials at extinction (day 2), f) mean percentage of time spent freezing during all 8 trials at extinction retrieval (day 3). °, $P < 0.05$ vs. CTRL-CTRL group. (N = 8 rats per group).

Discussion

The present findings indicate that repeated brief periods of SIS during early adolescence profoundly affect behavior later in life in a sex-dependent manner and that they

influence the ability to cope with a second challenge experienced later in life. Specifically, we showed that SIS induces in male adult rats hyperarousal and anxiety, without affecting locomotor activity and cognitive functions. Interestingly, in female adult rats exposed to SIS at adolescence only enhanced avoidance behavior was observed. We recently demonstrated that SPS experienced at adulthood induced a reduction of locomotor activity in both sexes, dysfunctions in spatial memory retention in males and altered fear memory dynamics selectively in females in the long-term (Mancini et al., 2020). When rats experienced SIS during early adolescence and then to SPS in adulthood resilience towards alterations in emotionality induced by SIS exposure in males and resilience towards SIS-induced avoidance behavior in females were observed. However, these effects seemed to be bidirectional. The SPS induced alterations of locomotor activity and spatial memory retention in males were not detected in rats that experienced SIS at adolescence.

It is well established that sociability represents a fundamental aspect during the adolescent period (Larson et al., 1996), thus problems with peer relationships, peer rejection or social deprivation may affect brain development resulting in an enhanced risk to develop psychopathologies later in life (Orben et al., 2020; Patterson, 1992). Similar to humans, compelling evidence has demonstrated that in rodents deprivation of social interactions with conspecifics, by using SIS paradigm especially during critical window for brain development, profoundly affects behavior in adulthood, resembling some of the core symptoms observed in schizophrenic patients (Fone and Porkess, 2008; Heidbreder et al., 2000; Mumtaz et al., 2018). However, the majority of studies using SIS models uses long and continuative protocol of this social stress thus lacking translational value. To our knowledge the present study provides the first evidence that brief and repeated periods of SIS experienced during early adolescence induce an anxious-like profile and hyperarousal later in life in male rats, as indicated by a reduced time spent in the center of the open field arena and in the open arms in the elevated plus maze, and by an enhanced startle response. This is consistent with previous studies demonstrating that long and continuative periods of SIS alter emotionality in male rats

in the long-term (Chappell et al., 2013; Lukkes et al., 2009b, 2009a; Skelly et al., 2015; Weiss et al., 2004; Wright et al., 1991). Zlatković and colleagues (2014) found that exposure to chronic SIS in adult rats increased avoidance behavior in a marble burying task (Zlatković et al., 2014). Conversely, here we found that repeated brief periods of SIS experienced during adolescence did not induce any effect in the marble burying task, suggesting that 2 hours of SIS for 7 consecutive days during early adolescence does not induce permanent effects on this behavioral domain. Further, we observed that brief and repeated periods of SIS in early adolescence does not affect locomotor activity in adult male rats. Literature data on SIS effects on rat locomotor behavior are often inconsistent (Walker et al., 2019). Even if several studies demonstrate hyperactivity in the open field task as a consequence of SIS (Archer, 1969; Dalrymple-Alford and Benton, 1981; Heidbreder et al., 2000; Skelly et al., 2015), few studies exist indicating hypoactivity (Holson et al., 1991, 1988) or no effects (Gardner et al., 1975; Thorsell et al., 2006; Weiss et al., 2004). Despite to previous studies showing that SIS for 6 hours per day from PND 15 to 21 enhanced spatial learning (Frisone et al., 2002) and that three weeks of SIS from PND 21 to 42 induced altered fear memory in adult male rats (Lukkes et al., 2009a), here we did not observe any SIS induced spatial or cued fear memory dysfunctions. Our results thus suggest that the effects induced by SIS could be age- and time- dependent indicating that brief and repeated periods of SIS from PND 28 to 34 do not induce cognitive alterations persisting in the long-term.

During adolescence components and mediators of the hypothalamic-pituitary-adrenal (HPA) axis, which is involved in the stress-response and in the regulation of coping strategies, are not fully mature yet, thus aversive stimuli, such as social stressors, may induce detrimental effects (Andersen, 2003; McCormick and Mathews, 2007; McEwen, 2007). It is well known that women a greater risk to develop stress-related disorders than men, and that this may in part depend from a dysregulation of the HPA axis (Bangasser and Valentino, 2014; Heck and Handa, 2019; McCormick and Mathews, 2007). Likewise, compelling evidence has demonstrated that sex differences in HPA function occur throughout this specific life stage. For example, adrenal volume

increases more in late adolescent females than in males (Green and McCormick, 2016; Heck and Handa, 2019; Pignatelli et al., 2006). The vast majority of the preclinical studies using SIS model have been carried out on males (Bagot et al., 2014) and only one study investigating the long-term effects induced by repeated brief periods of SIS in mid-adolescence period in females does exist (McCormick et al., 2010). Quite surprisingly, we found that 2 hours of SIS for 7 consecutive days during early adolescence do not alter the emotional profile in the elevated plus maze, the open field and the acoustic startle response tasks, but they enhanced avoidance and obsessive-compulsive behaviors in adult female rats in the marble burying task. Consistent with our findings, a previous study demonstrated that female adult rats exposed to early-life SIS did not exhibit an anxious-like phenotype compared to males (Weintraub et al., 2010), suggesting that probably the effects of adolescent SIS are more expressed in male rodents. We further showed that our model of SIS did not affect locomotor activity in females later in life. Results of the long-term consequences of SIS in females on locomotion are inconsistent (Walker et al., 2019), demonstrating in some cases hyperactivity (Dalrymple-Alford and Benton, 1981; Jahng et al., 2012), in others hypoactivity (Archer, 1969; Holson et al., 1991) or no effects (Archer, 1969; Butler et al., 2014). Additionally, we detected that brief and repeated periods of SIS do not produce any long-term effects on spatial memory and fear memory dynamics in females. Only one study evaluating the effects of long and continuative SIS in females on fear memory demonstrates less freezing in response to a conditioned tone compared to control females not exposed to stress (Weiss et al., 2004). Thus, in the light of our current results, it appears that brief and repeated SIS experienced during early adolescence does not alter fear memory processes.

According to the match/mismatch hypothesis, vulnerability or resilience to develope a psychopathology in adulthood depends by how early-life stressful events match the adversities later in life (Champagne et al., 2009; Daskalakis et al., 2013; Krugers et al., 2017; Santarelli et al., 2014). Growing evidence demonstrated that the adolescent deprivation of social interactions may enhance the risk to develop psychiatric disorders

later in life (Orben et al., 2020; Patterson, 1992). However, studies on the effects of the deprivation of social interactions during early adolescence on the vulnerability or resilience in response to a second challenge experienced later in life are sparse. Here, by using brief and repeated periods of SIS as a first stressor and an acute traumatic stress (SPS) as the second stressor, we assessed whether the interaction between SIS during early adolescence and SPS in adulthood alters the vulnerability or resilience towards stress-induced long-term emotional and cognitive functions. The SPS paradigm is a well-validated rodent model used to reproduce some of the hallmark symptoms observed in PTSD patients (e.g., hyperarousal, anxiety, and spatial and fear memory deficits) and the effects-induced require 7-14 days of incubation to develop (Iwamoto et al., 2007; Liberzon et al., 1997; Lisieski et al., 2018; Souza et al., 2017). We previously demonstrated that the SPS model is able to resemble the chronicity of PTSD-like symptomatology in a sex-dependent manner, with SPS-exposed males showing long-term reduced locomotion, hyperarousal, increased avoidance, anxiety-like behavior and spatial memory retention deficits and SPS-exposed females only presenting reduced locomotion but increased fear extinction 1 month after trauma (Mancini et al., 2020). It is important to note that in the present study we found that these SPS-induced effects did not persist when male rats were exposed to SIS during early adolescence, thus indicating a positive interaction between the two stressors with the social stress experienced during adolescence augmenting the ability of the rats to cope with additional trauma in adulthood, and thereby leading to increased resilience against stress-induced alterations. Interestingly, in females the avoidance and obsessive-compulsive like phenotype induced by SIS seems to disappear when rats were additionally exposed to a second challenge in adulthood. On the contrary, while the SPS-induced altered fear extinction was absent when rats were exposed to both stressors, the reduced locomotor activity is still persistent when female rats were previously exposed to SIS during early-adolescence, suggesting vulnerability towards altered locomotion induced by SPS.

This work is characterized by great translational value since nowadays adolescents

spend a large amount of time during the day playing video games and avoiding social relations. For instance, clinical studies showed that when adolescents spend a lot of time playing video games, they reduce time in interacting with friends or parents, participating in sports and other activities that are important for the development of their social skills (Cummings and Vandewater, 2007). This form of social isolation may induce several outcomes in adolescents such as social interaction problems, negative moods and depressive disorders (Zamani et al., 2010). Thus, it is very relevant to highlight that repeated brief periods of SIS per day during early adolescence could cause important long-term effects in a sex-divergent manner and that this brief and repeated model of SIS could alter resilience towards emotional and cognitive alterations when a traumatic event occurs later in life. Additional studies are needed to better understand the neurobiological mechanisms underlying such effects.

References

Almenrader, N., Colucci, P., De Castro, V., Valeri, D., Palmery, M., Trezza, V., Campolongo, P., 2017. Effects of sevoflurane and clonidine on acid base status and long-term emotional and cognitive outcomes in spontaneously breathing rat pups. PLoS One 12. https://doi.org/10.1371/journal.pone.0173969

Andersen, S.L., 2003. Trajectories of brain development: Point of vulnerability or window of opportunity?, in: Neuroscience and Biobehavioral Reviews. pp. 3–18. https://doi.org/10.1016/S0149-7634(03)00005-8

Anderson, C.A., Shibuya, A., Ihori, N., Swing, E.L., Bushman, B.J., Sakamoto, A., Rothstein, H.R., Saleem, M., 2010. Violent Video Game Effects on Aggression, Empathy, and Prosocial Behavior in Eastern and Western Countries: A Meta-Analytic Review. Psychol. Bull. https://doi.org/10.1037/a0018251

Andolina, D., Puglisi-Allegra, S., Ventura, R., 2015. Strain-dependent differences in corticolimbic processing of aversive or rewarding stimuli. Front. Syst. Neurosci. 8. https://doi.org/10.3389/fnsys.2014.00207

Archer, J., 1969. Contrasting effects of group housing and isolation on subsequent open field exploration in laboratory rats. Psychon. Sci. https://doi.org/10.3758/BF03332812

Atsak, P., Hauer, D., Campolongo, P., Schelling, G., McGaugh, J.L., Roozendaal, B., 2012. Glucocorticoids interact with the hippocampal endocannabinoid system in impairing retrieval of contextual fear memory. Proc. Natl. Acad. Sci. U. S. A. 109, 3504–3509.

https://doi.org/10.1073/pnas.1200742109
Bagot, R.C., Labonté, B., Peña, C.J., Nestler, E.J., 2014. Epigenetic signaling in psychiatric disorders: Stress and depression. Dialogues Clin. Neurosci. 16, 281–295.
Bangasser, D.A., Valentino, R.J., 2014. Sex differences in stress-related psychiatric disorders: Neurobiological perspectives. Front. Neuroendocrinol. https://doi.org/10.1016/j.yfrne.2014.03.008
Blakemore, S.J., Mills, K.L., 2014. Is adolescence a sensitive period for sociocultural processing? Annu. Rev. Psychol. https://doi.org/10.1146/annurev-psych-010213-115202
Blanchard, D.C., Blanchard, R.J., 1972. Innate and conditioned reactions to threat in rats with amygdaloid lesions. J. Comp. Physiol. Psychol. 81, 281–290. https://doi.org/10.1037/h0033521
Butler, T.R., Carter, E., Weiner, J.L., 2014. Adolescent Social Isolation Does Not Lead to Persistent Increases in Anxiety- Like Behavior or Ethanol Intake in Female Long-Evans Rats. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.12476
Butler, T.R., Karkhanis, A.N., Jones, S.R., Weiner, J.L., 2016. Adolescent Social Isolation as a Model of Heightened Vulnerability to Comorbid Alcoholism and Anxiety Disorders. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.13075
Champagne, D.L., Ronald de Kloet, E., Joëls, M., 2009. Fundamental aspects of the impact of glucocorticoids on the (immature) brain. Semin. Fetal Neonatal Med. 14, 136–142. https://doi.org/10.1016/j.siny.2008.11.006
Chappell, A.M., Carter, E., McCool, B.A., Weiner, J.L., 2013. Adolescent rearing conditions influence the relationship between initial anxiety-like behavior and ethanol drinking in male long evans rats. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.2012.01926.x
Colucci, P., Marchetta, E., Mancini, G.F., Alva, P., Chiarotti, F., Hasan, M.T., Campolongo, P., 2020. Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD). Transl. Psychiatry 10. https://doi.org/10.1038/s41398-020-00929-9
Cummings, H.M., Vandewater, E.A., 2007. Relation of adolescent video game play to time spent in other activities. Arch. Pediatr. Adolesc. Med. https://doi.org/10.1001/archpedi.161.7.684
Dalrymple-Alford, J.C., Benton, D., 1981. Activity differences of individually and group-housed male and female rats. Anim. Learn. Behav. https://doi.org/10.3758/BF03212025
Daskalakis, N.P., Bagot, R.C., Parker, K.J., Vinkers, C.H., de Kloet, E.R., 2013. The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome. Psychoneuroendocrinology 38, 1858–1873. https://doi.org/10.1016/j.psyneuen.2013.06.008
Daskalakis, N.P., Kloet, E.R. De, Yehuda, R., Malaspina, D., Kranz, T.M., 2015. Early life stress effects on glucocorticoid—BDNF interplay in the hippocampus. Front. Mol. Neurosci. https://doi.org/10.3389/fnmol.2015.00068
Faraji, J., Soltanpour, N., Ambeskovic, M., Zucchi, F.C.R., Beaumier, P., Kovalchuk, I., Metz, G.A.S., 2017. Evidence for ancestral programming of resilience in a two-hit stress model.

Front. Behav. Neurosci. 11. https://doi.org/10.3389/fnbeh.2017.00089

Fone, K.C.F., Porkess, M.V., 2008. Behavioural and neurochemical effects of post-weaning social isolation in rodents-Relevance to developmental neuropsychiatric disorders. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2008.03.003

Frisone, D.F., Frye, C.A., Zimmerberg, B., 2002. Social isolation stress during the third week of life has age-dependent effects on spatial learning in rats. Behav. Brain Res. https://doi.org/10.1016/S0166-4328(01)00315-1

Ganon-Elazar, E., Akirav, I., 2012a. Cannabinoids prevent the development of behavioral and endocrine alterations in a rat model of intense stress. Neuropsychopharmacology 37, 456–466. https://doi.org/10.1038/npp.2011.204

Ganon-Elazar, E., Akirav, I., 2012b. Cannabinoids prevent the development of behavioral and endocrine alterations in a rat model of intense stress. Neuropsychopharmacology 37, 456–466. https://doi.org/10.1038/npp.2011.204

Gardner, E.B., Boitano, J.J., Mancino, N.S., D'Amico, D.P., Gardner, E.L., 1975. Environmental enrichment and deprivation: Effects on learning, memory and exploration. Physiol. Behav. https://doi.org/10.1016/0031-9384(75)90040-2

Green, M.R., McCormick, C.M., 2016. Sex and stress steroids in adolescence: Gonadal regulation of the hypothalamic–pituitary–adrenal axis in the rat. Gen. Comp. Endocrinol. https://doi.org/10.1016/j.ygcen.2016.02.004

Han, R.T., Kim, Y.B., Park, E.H., Kim, J.Y., Ryu, C., Kim, H.Y., Lee, J.H., Pahk, K., Shanyu, C., Kim, H., Back, S.K., Kim, H.J., Kim, Y.I., Na, H.S., 2018. Long-Term Isolation Elicits Depression and Anxiety-Related Behaviors by Reducing Oxytocin-Induced GABAergic Transmission in Central Amygdala. Front. Mol. Neurosci. https://doi.org/10.3389/fnmol.2018.00246

Hankin, B.L., Abramson, L.Y., Moffitt, T.E., Angell, K.E., Silva, P.A., McGee, R., 1998. Development of depression from preadolescence to young adulthood: Emerging gender differences in a 10-year longitudinal study. J. Abnorm. Psychol. https://doi.org/10.1037/0021-843X.107.1.128

Heck, A.L., Handa, R.J., 2019. Sex differences in the hypothalamic–pituitary–adrenal axis' response to stress: an important role for gonadal hormones. Neuropsychopharmacology. https://doi.org/10.1038/s41386-018-0167-9

Heidbreder, C.A., Weiss, I.C., Domeney, A.M., Pryce, C., Homberg, J., Hedou, G., Feldon, J., Moran, M.C., Nelson, P., 2000. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neuroscience. https://doi.org/10.1016/S0306-4522(00)00336-5

Hill, R.A., Klug, M., Kiss Von Soly, S., Binder, M.D., Hannan, A.J., van Den Buuse, M., 2014. Sex-specific disruptions in spatial memory and anhedonia in a "two hit" rat model correspond with alterations in hippocampal brain-derived neurotrophic factor expression and signaling. Hippocampus 24, 1197–1211. https://doi.org/10.1002/hipo.22302

Holson, R.R., Scallet, A.C., Ali, S.F., Sullivan, P., Gough, B., 1988. Adrenocortical, β-endorphin and behavioral responses to graded stressors in differentially reared rats. Physiol. Behav.

https://doi.org/10.1016/0031-9384(88)90286-7
Holson, R.R., Scallet, A.C., Ali, S.F., Turner, B.B., 1991. “Isolation stress” revisited: Isolation-rearing effects depend on animal care methods. Physiol. Behav. https://doi.org/10.1016/0031-9384(91)90338-O
Ieraci, A., Mallei, A., Popoli, M., 2016. Social Isolation Stress Induces Anxious-Depressive-Like Behavior and Alterations of Neuroplasticity-Related Genes in Adult Male Mice. Neural Plast. https://doi.org/10.1155/2016/6212983
Iwamoto, Y., Morinobu, S., Takahashi, T., Yamawaki, S., 2007. Single prolonged stress increases contextual freezing and the expression of glycine transporter 1 and vesicle-associated membrane protein 2 mRNA in the hippocampus of rats. Prog. Neuro-Psychopharmacology Biol. Psychiatry 31, 642–651. https://doi.org/10.1016/j.pnpbp.2006.12.010
Jahng, J.W., Yoo, S.B., Ryu, V., Lee, J.H., 2012. Hyperphagia and depression-like behavior by adolescence social isolation in female rats. Int. J. Dev. Neurosci. https://doi.org/10.1016/j.ijdevneu.2011.10.001
Kohda, K., Harada, K., Kato, K., Hoshino, A., Motohashi, J., Yamaji, T., Morinobu, S., Matsuoka, N., Kato, N., 2007. Glucocorticoid receptor activation is involved in producing abnormal phenotypes of single-prolonged stress rats: A putative post-traumatic stress disorder model. Neuroscience 148, 22–33. https://doi.org/10.1016/j.neuroscience.2007.05.041
Krugers, H.J., Arp, J.M., Xiong, H., Kanatsou, S., Lesuis, S.L., Korosi, A., Joels, M., Lucassen, P.J., 2017. Early life adversity: Lasting consequences for emotional learning. Neurobiol. Stress. https://doi.org/10.1016/j.ynstr.2016.11.005
Lapiz, M.D.S., Fulford, A., Muchimapura, S., Mason, R., Parker, T., Marsden, C.A., 2003. Influence of postweaning social isolation in the rat on brain development, contioned behavior, and neurotransmission. Neurosci. Behav. Physiol. https://doi.org/10.1023/A:1021171129766
Larson, R.W., Moneta, G., Richards, M.H., Holmbeck, G., Duckett, E., 1996. Changes in adolescents’ daily interactions with their families from ages 10 to 18: Disengagement and transformation. Dev. Psychol. https://doi.org/10.1037/0012-1649.32.4.744
Liberzon, I., Krstov, M., Young, E.A., 1997. Stress-restress: Effects on ACTH and fast feedback. Psychoneuroendocrinology 22, 443–453. https://doi.org/10.1016/S0306-4530(97)00044-9
Liberzon, I., López, J.F., Flagel, S.B., Vázquez, D.M., Young, E.A., 1999. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: Relevance to post-traumatic stress disorder. J. Neuroendocrinol. https://doi.org/10.1046/j.1365-2826.1999.00288.x
Lisieski, M.J., Eagle, A.L., Conti, A.C., Liberzon, I., Perrine, S.A., 2018. Single-prolonged stress: A review of two decades of progress in a rodent model of post-traumatic stress disorder. Front. Psychiatry. https://doi.org/10.3389/fpsyt.2018.00196
Lukkes, J.L., Mokin, M. V., Scholl, J.L., Forster, G.L., 2009a. Adult rats exposed to early-life social isolation exhibit increased anxiety and conditioned fear behavior, and altered hormonal stress responses. Horm. Behav. https://doi.org/10.1016/j.yhbeh.2008.10.014

Lukkes, J.L., Watt, M.J., Lowry, C.A., Forster, G.L., 2009b. Consequences of post-weaning social isolation on anxiety behavior and related neural circuits in rodents. Front. Behav. Neurosci. https://doi.org/10.3389/neuro.08.018.2009

Mancini, G.F., Marchetta E., Riccardi E., Trezza V., Morena M., Campolongo P., 2020. Sex-divergent long-term effects of single prolonged stress in adult rats. Behav. Brain Res. https://doi.org/ 10.1016/j.bbr.2020.113096

McCormick, C.M., Mathews, I.Z., 2007. HPA function in adolescence: Role of sex hormones in its regulation and the enduring consequences of exposure to stressors. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2006.07.012

McCormick, C.M., Nixon, F., Thomas, C., Lowie, B., Dyck, J., 2010. Hippocampal cell proliferation and spatial memory performance after social instability stress in adolescence in female rats. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2009.11.003

McCormick, C.M., Robarts, D., Kopeikina, K., Kelsey, J.E., 2005. Long-lasting, sex- and age-specific effects of social stressors on corticosterone responses to restraint and on locomotor responses to psychostimulants in rats. Horm. Behav. https://doi.org/10.1016/j.yhbeh.2005.01.008

McEwen, B.S., 2016. In pursuit of resilience: stress, epigenetics, and brain plasticity. Ann. N. Y. Acad. Sci. https://doi.org/10.1111/nyas.13020

McEwen, B.S., 2007. Physiology and neurobiology of stress and adaptation: Central role of the brain. Physiol. Rev. https://doi.org/10.1152/physrev.00041.2006

Medendorp, W.E., Petersen, E.D., Pal, A., Wagner, L.M., Myers, A.R., Hochgeschwender, U., Jenrow, K.A., 2018. Altered behavior in mice socially isolated during adolescence corresponds with immature dendritic spine morphology and impaired plasticity in the prefrontal cortex. Front. Behav. Neurosci. https://doi.org/10.3389/fnbeh.2018.00087

Mihara, S., Higuchi, S., 2017. Cross-sectional and longitudinal epidemiological studies of Internet gaming disorder: A systematic review of the literature. Psychiatry Clin. Neurosci. https://doi.org/10.1111/pcn.12532

Morena, M., De Castro, V., Gray, J.M., Palmery, M., Trezza, V., Roozendaal, B., Hill, M.N., Campolongo, P., 2015. Training-associated emotional arousal shapes endocannabinoid modulation of spatial memory retrieval in rats. J. Neurosci. 35, 13962–13974. https://doi.org/10.1523/JNEUROSCI.1983-15.2015

Morena, M., Leitl, K.D., Vecchiarelli, H.A., Gray, J.M., Campolongo, P., Hill, M.N., 2016. Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety. Neuropharmacology 111, 59–69. https://doi.org/10.1016/j.neuropharm.2016.08.020

Müller, K.W., Beutel, M.E., Egloff, B., Wölfling, K., 2014. Investigating risk factors for internet gaming disorder: A comparison of patients with addictive gaming, pathological gamblers and healthy controls regarding the big five personality traits. Eur. Addict. Res. https://doi.org/10.1159/000355832

Mumtaz, F., Khan, M.I., Zubair, M., Dehpour, A.R., 2018. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. Biomed. Pharmacother.

https://doi.org/10.1016/j.biopha.2018.05.086
Orben, A., Tomova, L., Blakemore, S.J., 2020. The effects of social deprivation on adolescent development and mental health. Lancet Child Adolesc. Heal. https://doi.org/10.1016/S2352-4642(20)30186-3
Patterson, G.R., 1992. Developmental Changes in Antisocial Behaviour, in: Aggression and Violence Throughout the Life Span.
Patterson, T.L., Smith, L.W., Smith, T.L., Yager, J., Grant, I., 1992. Symptoms of illness in late adulthood are related to childhood social deprivation and misfortune in men but not in women. J. Behav. Med. https://doi.org/10.1007/BF00848320
Peña, C.J., Kronman, H.G., Walker, D.M., Cates, H.M., Bagot, R.C., Purushothaman, I., Issler, O., Eddie Loh, Y.H., Leong, T., Kiraly, D.D., Goodman, E., Neve, R.L., Shen, L., Nestler, E.J., 2017. Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. Science (80-.). 356, 1185–1188. https://doi.org/10.1126/science.aan4491
Pignatelli, D., Xiao, F., Gouveia, A.M., Ferreira, J.G., Vinson, G.P., 2006. Adrenarche in the rat. J. Endocrinol. https://doi.org/10.1677/joe.1.06972
Rodriguez-Romaguera, J., Sotres-Bayon, F., Mueller, D., Quirk, G.J., 2009. Systemic Propranolol Acts Centrally to Reduce Conditioned Fear in Rats Without Impairing Extinction. Biol. Psychiatry 65, 887–892. https://doi.org/10.1016/j.biopsych.2009.01.009
Santarelli, S., Lesuis, S.L., Wang, X.D., Wagner, K. V., Hartmann, J., Labermaier, C., Scharf, S.H., Müller, M.B., Holsboer, F., Schmidt, M. V., 2014. Evidence supporting the match/mismatch hypothesis of psychiatric disorders. Eur. Neuropsychopharmacol. 24, 907–918. https://doi.org/10.1016/j.euroneuro.2014.02.002
Scuderi, C., Stecca, C., Valenza, M., Ratano, P., Bronzuoli, M.R., Bartoli, S., Steardo, L., Pompili, E., Fumagalli, L., Campolongo, P., Steardo, L., 2014. Palmitoylethanolamide controls reactive gliosis and exerts neuroprotective functions in a rat model of Alzheimer's disease. Cell Death Dis. 5. https://doi.org/10.1038/cddis.2014.376
Shetty, R.A., Sadananda, M., 2017. Brief Social Isolation in the Adolescent Wistar-Kyoto Rat Model of Endogenous Depression Alters Corticosterone and Regional Monoamine Concentrations. Neurochem. Res. https://doi.org/10.1007/s11064-017-2203-2
Skelly, M.J., Chappell, A.E., Carter, E., Weiner, J.L., 2015. Adolescent social isolation increases anxiety-like behavior and ethanol intake and impairs fear extinction in adulthood: Possible role of disrupted noradrenergic signaling. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2015.05.025
Souza, R.R., Noble, L.J., McIntyre, C.K., 2017. Using the single prolonged stress model to examine the pathophysiology of PTSD. Front. Pharmacol. 8. https://doi.org/10.3389/fphar.2017.00615
Spear, L.P., 2000. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev. 24, 417–463. https://doi.org/10.1016/S0149-7634(00)00014-2
Thorsell, A., Slawecki, C.J., El Khoury, A., Mathe, A.A., Ehlers, C.L., 2006. The effects of social isolation on neuropeptide Y levels, exploratory and anxiety-related behaviors in rats.

Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2005.12.005

Trezza, V., Baarendse, P.J.J., Vanderschuren, L.J.M.J., 2010. The pleasures of play: Pharmacological insights into social reward mechanisms. Trends Pharmacol. Sci. https://doi.org/10.1016/j.tips.2010.06.008

Trezza, V., Campolongo, P., Vanderschuren, L.J.M.J., 2011. Evaluating the rewarding nature of social interactions in laboratory animals. Dev. Cogn. Neurosci. https://doi.org/10.1016/j.dcn.2011.05.007

Tsoory, M., Cohen, H., Richter-Levin, G., 2007. Juvenile stress induces a predisposition to either anxiety or depressive-like symptoms following stress in adulthood. Eur. Neuropsychopharmacol. https://doi.org/10.1016/j.euroneuro.2006.06.007

Von Der Heiden, J.M., Braun, B., Müller, K.W., Egloff, B., 2019. The association between video gaming and psychological functioning. Front. Psychol. https://doi.org/10.3389/fpsyg.2019.01731

Walker, D.M., Cunningham, A.M., Gregory, J.K., Nestler, E.J., 2019. Long-term behavioral effects of post-weaning social isolation in males and females. Front. Behav. Neurosci. https://doi.org/10.3389/fnbeh.2019.00066

Weintraub, A., Singaravelu, J., Bhatnagar, S., 2010. Enduring and sex-specific effects of adolescent social isolation in rats on adult stress reactivity. Brain Res. https://doi.org/10.1016/j.brainres.2010.04.068

Weiss, I.C., Pryce, C.R., Jongen-Rêlo, A.L., Nanz-Bahr, N.I., Feldon, J., 2004. Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2003.10.015

Wright, I.K., Upton, N., Marsden, C.A., 1991. Resocialisation of isolation-reared rats does not alter their anxiogenic profile on the elevated X-maze model of anxiety. Physiol. Behav. https://doi.org/10.1016/0031-9384(91)90572-6

Zamani, E., Kheradmand A., Cheshmi M., Abedi A., Hedayati N., 2010. Comparing the social skills of students addicted to computer games with normals students. Addict Health. 2(3-4):59-65

Zanda, M.T., Fadda, P., Antinori, S., Di Chio, M., Fratta, W., Chiamulera, C., Fattore, L., 2017. Methoxetamine affects brain processing involved in emotional response in rats. Br. J. Pharmacol. 174, 3333–3345. https://doi.org/10.1111/bph.13952

Zlatković, J., Todorović, N., Bošković, M., Pajović, S.B., Demajo, M., Filipović, D., 2014. Different susceptibility of prefrontal cortex and hippocampus to oxidative stress following chronic social isolation stress. Mol. Cell. Biochem. https://doi.org/10.1007/s11010-014-2045-z

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Stressful experiences can produce both accurate or generalized memories, reflecting an interindividual difference in response to stress (Bahtiyar et al., 2020; Kalisch et al., 2017; Wu et al., 2013). It has long been observed in humans that the intake of drugs of abuse affects memory processes (Goodman and Packard, 2016; Kutlu and Gould, 2016), but their effects on memory generalization are poor investigated. The enhanced memory consolidation may lead to the excessive memory retrieval in a situational reminder condition leading to memory generalization, which is one of the symptoms of PTSD (American Psychiatric Association, 2013; Yehuda et al., 2015). Notwithstanding, the memory enhancing effects of amphetamine are well-established, currently, its effect on memory generalization is poor investigated. Moreover, we recently demonstrated that the 3,4-methylenedioxypyrovalerone (MDPV), a newer synthetic cathinone also known as ‘‘bath salt,’’ enhances short-term spatial and recognition memory performance (Atehortua-Martinez et al., 2019). However, still MDPV effects on memory generalization have not yet been studied. Thus, in **Chapter 1** we aimed at investigating the memory generalization effects induced by a known consolidation enhancer, such as amphetamine, and a new psychostimulant MDPV. Our results indicate that both amphetamine and MDPV induce generalization of fear memory, but only amphetamine enhances memory consolidation. Since both amphetamine and MDPV modulate noradrenaline and dopamine tone, we also aimed at evaluating the involvement of the noradrenergic and dopaminergic systems in mediating their effects on fear memory generalization. Our results indicate that the noradrenergic system is responsible for the memory consolidation enhancement exerted by amphetamine. Of novel interest, we demonstrated that the noradrenergic

system also modulates the generalization effects induced by both amphetamine and MDPV. In particular, our findings indicate that amphetamine effects on memory generalization are partially blocked by preventive administration of the β-adrenoceptor antagonist propranolol, while MDPV effects are totally blocked by propranolol administration. Conversely, we found that the generalization effect induced by amphetamine is strongly regulated by the dopaminergic system, whereas the MDPV effects on memory generalization are due to a selective activation of the noradrenergic system. Although these results require further investigation, it can be hypothesized that these memory generalization effects are due to a differential recruitment induced by amphetamine and MDPV on the monoamine systems in different brain areas particularly involved in memory generalization, such as medial prefrontal cortex, nucleus reunions, and hippocampus (Xu and Südhof, 2013).

Treatment with the anesthetic ketamine (a renowned drug of abuse) in trauma patients during emergency care, presents aggravated early post- traumatic stress reaction which is highly predictive of PTSD development and severity (Schönenberg et al., 2008, 2005; Winter and Irle, 2004). Based on the evidence that ketamine induces a robust central and peripheral adrenergic/noradrenergic potentiation, and that activation of this system is essential for the formation of memory for stressful events, in **Chapter 2** we explored the possibility that the strong sympathomimetic action of ketamine might underlie its memory enhancing effects. Our results reveal that ketamine anesthesia modulates time-dependent processes of aversive memory consolidation together with a combined peripheral and central action of ketamine in potentiating adrenergic/noradrenergic signaling to promote aversive memory retention. Adrenal medullectomy or blockade of ß-adrenoceptors, with systemic injections of propranolol, although not altering 48-h memory retention *per se*, prevented the memory potentiating effects of immediate post-training ketamine injection. Furthermore, our findings show that temporary post-training lesions of the locus coerules completely blocked the memory enhancing effects of ketamine. Our last

experiment identified the basolateral amygdala as a candidate brain area responsible for ketamine-mediated enhancement of traumatic memory retention. Selective blockade of ß-adrenoceptors within the basolateral amygdala completely prevented the effects of post-training ketamine anesthesia. Given that PTSD is a highly debilitating psychiatric disorder and very difficult to treat, our findings present a strong translational value, as they have the potential to inform clinicians for a better management of ketamine anesthesia in emergency care and trauma victims.

Since PTSD is a chronic psychiatric disease, it is thus of critical importance to evaluate whether animal models of PTSD are able to resemble the chronicity nature of this pathology. The single prolonged stress paradigm has been extensively shown to induce behavioral and endocrine effects resembling some of the hallmark symptoms observed in PTSD patients (Lisieski et al., 2018; Souza et al., 2017; Verbitsky et al., 2020; Yamamoto et al., 2009); the manifestation of these effects is time-dependent and requires a 1-2 weeks incubation period (Knox et al., 2012; Liberzon et al., 1999; Wu et al., 2016). In **Chapter 3** we aimed at investigating whether single prolonged stress induced persistent PTSD-like behavioral alterations in rats long after trauma exposure and whether these effects are sex-dependent. To this aim, separate cohorts of male and female adult rats were subjected to single prolonged stress and, 30 days later, long-term effects were assessed. We found that single prolonged stress exposure reduced locomotor activity in both sexes in an open field task. Males only showed increased anxiety-like behavior in the elevated plus maze and marble burying tests, enhanced acoustic startle response and impaired spatial memory retention while females were unaffected. The single prolonged stress exposure did not alter auditory fear memory dynamics in males, but it did alter extinction retrieval in females. We provide the first evidence that single prolonged stress reproduces long-term emotional alterations in male, but not in female, rats which persist 30 days following trauma exposure, thus resembling some of the hallmark symptoms of PTSD. Our results provide evidence of fundamental sex-differences in the response to a traumatic event

in rats and the subsequent susceptibility to develop a PTSD-like symptomatology. Thus, our findings are relevant to future research aimed not only at investigating sex-differences in the neurobiology of trauma-related disorders, but also at evaluating pharmacological interventions to treat long-lasting emotional alterations associated with PTSD.

As we mention before, it is also important to take in consideration the susceptibility aspect of PTSD. Animal models are a useful tool to investigate this issue (Musazzi et al., 2018). In **Chapter 4** we aimed at the development of an animal model of PTSD able to predict the susceptibility and the resilience phenotype, with a high translational value to human PTSD, considering both cognitive and emotional alterations long after trauma. For this purpose, we outstretched our previously validated animal model (Berardi et al., 2014) in order to identify before the extinction sessions and drug treatment, susceptible and resilient rats in terms of over- consolidation, impaired extinction and social behavior alterations. We identified a predictive variable for the screening of PTSD susceptibility and resilience in term of over-consolidation, impaired extinction, and social impairment. Particularly, we found as a reliable predictive variable the number of crossings (locomotor activity) in the Open Field test. The analysis of the number of crossings in the Open Field test is an easy and valuable tool to study at the same time the motor activity and the natural tendency of rodents to explore a new environment (Denenberg, 1969). Here we further dissociated these two aspects, in order to better understand if it is the motor activity *per se*, or the natural tendency to explore a new environment that made the number of crossings a reliable predictive variable for the development of PTSD-like phenotype. Our results clearly indicate that the changes induced by trauma exposure on the number of crossings make it a predictive variable for susceptibility and resilience towards developing a PTSD-like phenotype.

Nowadays there are different hypotheses to explain the interindividual variability in

response to stress. Different studies used the "two hit" stress model to investigate whether exposure to two different stressors at different ages may increase (or decrease) the risk to develop psychopathologies after experiencing the second stressor (Hill et al., 2014; Peña et al., 2017; Tsoory et al., 2007). However, how a social stress similar to bullying in humans experienced at adolescence may affect the reaction to additional stressor later in life are less investigated (Buwalda et al., 2013). Adolescence is a period of impressive brain maturation in which the structure of the brain is ever-changing (Spear et al., 2000). Thus, maintaining a correct balance between mediators that sustain synaptic plasticity is of utmost importance. In **Chapter 5** we evaluated whether exposure to social defeat stress, a highly validated animal model of bullying in rodents, at adolescence and/or single prolonged stress experienced at adulthood affect the later development of emotional and cognitive alterations and whether the behavioral alterations are linked to any modification of hippocampal brain derived neurotrophic factor (BDNF) expression and plasma corticosterone levels. The present findings indicate that exposure to social stress during early adolescence influences the ability to cope with a second challenge experienced later in life. Specifically, rats exposed to social defeat stress during early adolescence and then to single prolonged stress in adulthood exhibited resilience against the development of alterations in emotionality, arousal, and spatial memory, but demonstrated vulnerability toward cued fear memory dysfunction. In addition, rats exposed to both stressors showed resilience toward alterations induced by social defeat stress exposure on the hippocampal BDNF protein expression and plasma corticosterone levels. These effects seemed to be bidirectional; SPS alone induced alterations of locomotor activity and spatial memory retention in the long-term (Mancini et al., 2020) that were not detected in rats that experienced social defeat stress during adolescence. To the best of our knowledge, this study is the first to evaluate whether exposure to an early-life stress protocol that mimics bullying in adolescents affects the development of psychopathologies in adulthood in response to additional trauma experienced later in life. These results pave the way for future studies aimed at investigating the role of

specific brain areas and neurotransmission in mediating the mechanisms that confer resilience or vulnerability to early-life stress exposure.

While adolescent bullying occurs in a small subset of population, a more common social stressor is represented by the deprivation of social interactions with conspecifics which is able to induce profound behavioral changes in rodents (Fone and Porkess, 2008; Heidbreder et al., 2000). Social isolation stress is a well-validated stress paradigm used to reproduce in rodents alterations resembling those of different psychiatric disorders. SIS is generally performed in male rats starting from adolescence until adulthood for long and continuative periods (Fone and Porkess, 2008; Mumtaz et al., 2018). However, the long-term effects of a briefer period of SIS occurring only during early adolescence and potential sex-differences in response to SIS are not investigated. Moreover, early-life adverse experiences (first hit) may affect resilience or vulnerability to later experienced stressful events (second hit) and the subsequent development of stress-related psychopathologies. In **Chapter 6** we firstly evaluated whether repeated brief periods of social isolation stress may alter emotionality and cognitive function in adult rats and whether these effects are sex-dependent. Secondly, we examined whether brief and repeated social isolation stress during adolescence and/or single prolonged stress at adulthood affect the later development of alterations on emotionality and cognition. We found that social isolation stress induces hyperarousal and anxious-like phenotype only in male adult rats, and enhanced avoidance behavior in female adult rats. As we demonstrated, SPS induced a reduction of locomotor activity in both sexes, dysfunctions in spatial memory retention in males and altered fear memory dynamics in females (Mancini et al., 2020). Rats experienced both stressors exhibited resilience towards alterations in emotionality induced by social isolation stress exposure in males and towards SIS-induced avoidance behavior in females. The single prolonged stress altered locomotion and spatial memory retention; these effects were absent in rats exposed to

social isolation stress and later exposed to single prolonged stress. This work is characterized by great translational value since nowadays adolescents spend a large amount of time during the day playing video games and avoiding social relations. For instance, clinical studies showed that when adolescents spend a lot of time playing video games, they reduce time in interacting with friends or parents, participating in sports and other activities that are important for the development of their social skills (Cummings and Vandewater, 2007). This form of social isolation may induce several outcomes in adolescents such as social interaction problems, negative moods and depressive disorders (Zamani et al., 2010). Thus, it is very relevant to highlight that repeated brief periods of social isolation stress per day during early adolescence could cause important long-term effects in a sex-divergent manner and that this brief and repeated model of social isolation stress could alter resilience towards emotional and cognitive alterations when a traumatic event occurs later in life. Additional studies are needed to better understand the neurobiological mechanisms underlying such effects.

All together our results provide new insights in the susceptibility and resilience mechanisms. Particularly, investigating how an early-life social stress could change the individual responsiveness to an additional trauma experienced later in life, and sex differences involved in these processes, could lead to important advances in daily clinical practices, particularly in treating stress related disorders such as PTSD.

References

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association. https://doi.org/10.1176/appi.books.9780890425596

Atehortua-Martinez, L.A., Masniere, C., Campolongo, P., Chasseigneaux, S., Callebert, J., Zwergel, C., Mai, A., Laplanche, J.L., Chen, H., Etheve-Quelquejeu, M., Mégarbane, B., Benturquia, N., 2019. Acute and chronic neurobehavioral effects of the designer drug and bath salt constituent 3,4-methylenedioxypyrovalerone in the rat. J. Psychopharmacol. https://doi.org/10.1177/0269881118822151

Bahtiyar, S., Gulmez Karaca, K., Henckens, M.J.A.G., Roozendaal, B., 2020. Norepinephrine

and glucocorticoid effects on the brain mechanisms underlying memory accuracy and generalization. Mol. Cell. Neurosci. https://doi.org/10.1016/j.mcn.2020.103537

Berardi, A., Trezza, V., Palmery, M., Trabace, L., Cuomo, V., Campolongo, P., 2014. An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD). Front. Behav. Neurosci. 8. https://doi.org/10.3389/fnbeh.2014.00142

Buwalda, B., Stubbendorff, C., Zickert, N., Koolhaas, J.M., 2013. Adolescent social stress does not necessarily lead to a compromised adaptive capacity during adulthood: A study on the consequences of social stress in rats. Neuroscience. https://doi.org/10.1016/j.neuroscience.2012.12.050

Cummings, H.M., Vandewater, E.A., 2007. Relation of adolescent video game play to time spent in other activities. Arch. Pediatr. Adolesc. Med. https://doi.org/10.1001/archpedi.161.7.684

Denenberg, V.H., 1969. OPEN-FIELD BEHAVIOR IN THE RAT: WHAT DOES IT MEAN? Ann. N. Y. Acad. Sci. https://doi.org/10.1111/j.1749-6632.1969.tb12983.x

Fone, K.C.F., Porkess, M.V., 2008. Behavioural and neurochemical effects of post-weaning social isolation in rodents-Relevance to developmental neuropsychiatric disorders. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2008.03.003

Goodman, J., Packard, M.G., 2016. Memory systems and the addicted brain. Front. Psychiatry. https://doi.org/10.3389/fpsyt.2016.00024

Heidbreder, C.A., Weiss, I.C., Domeney, A.M., Pryce, C., Homberg, J., Hedou, G., Feldon, J., Moran, M.C., Nelson, P., 2000. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neuroscience. https://doi.org/10.1016/S0306-4522(00)00336-5

Hill, R.A., Kiss Von Soly, S., Ratnayake, U., Klug, M., Binder, M.D., Hannan, A.J., van den Buuse, M., 2014. Long-term effects of combined neonatal and adolescent stress on brain-derived neurotrophic factor and dopamine receptor expression in the rat forebrain. Biochim. Biophys. Acta - Mol. Basis Dis. 1842, 2126–2135. https://doi.org/10.1016/j.bbadis.2014.08.009

Kalisch, R., Baker, D.G., Basten, U., Boks, M.P., Bonanno, G.A., Brummelman, E., Chmitorz, A., Fernàndez, G., Fiebach, C.J., Galatzer-Levy, I., Geuze, E., Groppa, S., Helmreich, I., Hendler, T., Hermans, E.J., Jovanovic, T., Kubiak, T., Lieb, K., Lutz, B., Müller, M.B., Murray, R.J., Nievergelt, C.M., Reif, A., Roelofs, K., Rutten, B.P.F., Sander, D., Schick, A., Tüscher, O., Van Diest, I., Van Harmelen, A.L., Veer, I.M., Vermetten, E., Vinkers, C.H., Wager, T.D., Walter, H., Wessa, M., Wibral, M., Kleim, B., 2017. The resilience framework as a strategy to combat stress-related disorders. Nat. Hum. Behav. https://doi.org/10.1038/s41562-017-0200-8

Knox, D., George, S.A., Fitzpatrick, C.J., Rabinak, C.A., Maren, S., Liberzon, I., 2012. Single prolonged stress disrupts retention of extinguished fear in rats. Learn. Mem. https://doi.org/10.1101/lm.024356.111

Kutlu, M.G., Gould, T.J., 2016. Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: Contributions to development and maintenance of addiction. Learn. Mem. https://doi.org/10.1101/lm.042192.116

Liberzon, I., López, J.F., Flagel, S.B., Vázquez, D.M., Young, E.A., 1999. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: Relevance to post-

traumatic stress disorder. J. Neuroendocrinol. 11, 11–17. https://doi.org/10.1046/j.1365-2826.1999.00288.x

Lisieski, M.J., Eagle, A.L., Conti, A.C., Liberzon, I., Perrine, S.A., 2018. Single-prolonged stress: A review of two decades of progress in a rodent model of post-traumatic stress disorder. Front. Psychiatry. https://doi.org/10.3389/fpsyt.2018.00196

Mancini G.F.; Marchetta E.; Riccardi E.; Trezza V.; Morena M.; Campolongo P. Sex-divergent long-term effects of single prolonged stress in adult rats. Behav. Brain Res. 2020, 113096. doi: 10.1016/j.bbr.2020.113096.

Mumtaz, F., Khan, M.I., Zubair, M., Dehpour, A.R., 2018. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. Biomed. Pharmacother. https://doi.org/10.1016/j.biopha.2018.05.086

Musazzi, L., Tornese, P., Sala, N., Popoli, M., 2018. What acute stress protocols can tell us about PTSD and stress-related neuropsychiatric disorders. Front. Pharmacol. https://doi.org/10.3389/fphar.2018.00758

Peña, C.J., Kronman, H.G., Walker, D.M., Cates, H.M., Bagot, R.C., Purushothaman, I., Issler, O., Eddie Loh, Y.H., Leong, T., Kiraly, D.D., Goodman, E., Neve, R.L., Shen, L., Nestler, E.J., 2017. Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. Science (80-.). 356, 1185–1188. https://doi.org/10.1126/science.aan4491

Schönenberg, M., Reichwald, U., Domes, G., Badke, A., Hautzinger, M., 2008. Ketamine aggravates symptoms of acute stress disorder in a naturalistic sample of accident victims. J. Psychopharmacol. 22, 493–497. https://doi.org/10.1177/0269881107082481

Schönenberg, M., Reichwald, U., Domes, G., Badke, A., Hautzinger, M., 2005. Effects of peritraumatic ketamine medication on early and sustained posttraumatic stress symptoms in moderately injured accident victims. Psychopharmacology (Berl). 182, 420–5. https://doi.org/10.1007/s00213-005-0094-4

Souza, R.R., Noble, L.J., McIntyre, C.K., 2017. Using the single prolonged stress model to examine the pathophysiology of PTSD. Front. Pharmacol. 8. https://doi.org/10.3389/fphar.2017.00615

Spear, L.P., 2000. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev. https://doi.org/10.1016/S0149-7634(00)00014-2

Tsoory, M., Cohen, H., Richter-Levin, G., 2007. Juvenile stress induces a predisposition to either anxiety or depressive-like symptoms following stress in adulthood. Eur. Neuropsychopharmacol. 17, 245–256. https://doi.org/10.1016/j.euroneuro.2006.06.007

Verbitsky, A., Dopfel, D., Zhang, N., 2020. Rodent models of post-traumatic stress disorder: behavioral assessment. Transl. Psychiatry. https://doi.org/10.1038/s41398-020-0806-x

Winter, H., Irle, E., 2004. Hippocampal volume in adult burn patients with and without posttraumatic stress disorder. Am. J. Psychiatry 161, 2194–200. https://doi.org/10.1176/appi.ajp.161.12.2194

Wu, G., Feder, A., Cohen, H., Kim, J.J., Calderon, S., Charney, D.S., Mathé, A.A., 2013. Understanding resilience. Front. Behav. Neurosci. https://doi.org/10.3389/fnbeh.2013.00010

Wu, Z., Tian, Q., Li, F., Gao, J., Liu, Y., Mao, M., Liu, J., Wang, S., Li, G., Ge, D., Mao, Y.,

Zhang, W., Liu, Z., Song, Y., 2016. Behavioral changes over time in post-traumatic stress disorder: Insights from a rat model of single prolonged stress. Behav. Processes. https://doi.org/10.1016/j.beproc.2016.01.001

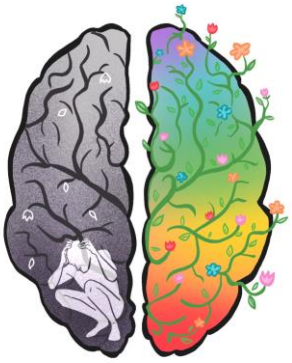
Xu, W., Südhof, T.C., 2013. A neural circuit for memory specificity and generalization. Science (80-.). https://doi.org/10.1126/science.1229534

Yamamoto, S., Morinobu, S., Takei, S., Fuchikami, M., Matsuki, A., Yamawaki, S., Liberzon, I., 2009. Single prolonged stress: Toward an animal model of posttraumatic stress disorder. Depress. Anxiety. https://doi.org/10.1002/da.20629

Yehuda, R., Hoge, C.W., McFarlane, A.C., Vermetten, E., Lanius, R.A., Nievergelt, C.M., Hobfoll, S.E., Koenen, K.C., Neylan, T.C., Hyman, S.E., 2015. Post-traumatic stress disorder. Nat. Rev. Dis. Prim. https://doi.org/10.1038/nrdp.2015.57

Zamani, E., Kheradmand A., Cheshmi M., Abedi A., Hedayati N., 2010. Comparing the social skills of students addicted to computer games with normals students. Addict Health. 2(3-4):59-65.

CURRICULUM VITAE



PERSONAL INFORMATION

Name:	**Giulia Federica Mancini**
Date and place of birth	June 23rd, 1993, San Benedetto del Tronto (Italy)
Nationality:	Italian

EDUCATION AND TRAINING

Sept 2019	**Course on Learning and Memory.** Neuroscience School of Advanced Studies (NSAS).
Dec 2017	**Board-Certified Pharmacist Doctor (PharmD).** Sapienza University of Rome, Italy.
Nov 2017-Dec 2020	**PhD student** in "Pharmacology and Toxicology". Dept. of Physiology and Pharmacology "V.Erspamer". Sapienza University of Rome, Italy.
Oct 2017	**Master of Pharmacy** (final marks: 110/110 cum laude). Sapienza Univ. of Rome, Italy. An experimental dissertation titled: "*Amphetamine and the 'bath salt' 3,4-methylenedyoxypyrovalerone (MDPV) differentially affect the accuracy of memory*".
Jul 2017-Sept 2017	**Visiting student**, Faculty of Basic and Biomedical Sciences, Paris Descartes University, Paris, (France).
Oct 2016-Oct 2017	**Master thesis.** Dept. of Physiology and Pharmacology, Sapienza University of Rome, Italy. Supervisor: Prof. Patrizia Campolongo.

PRIZES/AWARDS/FELLOWSHIPS

2020 Research fellowship released by Fondazione Santa Lucia, Italy (Jan 2021-March 2021).

2019 ISPNE Travel Award to support the attendance to the International Society of Psychoneuroendocrinology (ISPNE) Conference, 29-31 August 2019, Milan, Italy.

2019 Best poster prize. Poster titled: "Long-term behavioral effects of brief and repeated periods of social isolation stress during early-adolescence", Mediterranean Neuroscience Society 7^{rh} Conference 23-27 June 2019, Marrakech, Morocco.

2017 Individual three years PhD fellowship (Ministry of Education, Italy).

2017 Fellowship for neuroscience project abroad released by Associazione Valentina de Castro, Italy.

2017 Fellowship for thesis abroad released by Sapienza University of Rome, Italy.

RESEARCH SUPPORT AS PARTICIPANT

2018-2021 Sapienza Università di Roma (27.000 €). Research project title "Enhancing strength and generalization of extinction memory to treat post-traumatic stress disorder (PTSD): a preclinical study focusing on the interplay between the glucocorticoid and endocannabinoid systems".

2017-2020 Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale (263.977 €). Research project title "Early life stress and psychopathology: unravelling the mechanisms of vulnerability and resilience".

2017-2020 ERA-NET NEURON (250.000 €). Research project title "Mapping and interrogating top-down control of the memory engram of the posttraumatic stress disorder".

RESEARCH SUPPORT AS PARTICIPANT

2020 Grant "Avvio alla Ricerca 2020" released by the Sapienza University of Rome, Italy. Research project title "Isolamento sociale breve e ripetuto in adolescenza: meccanismi neurobiologici di suscettibilità e resilienza verso lo sviluppo di patologie psichiatriche in ratti maschi e femmina adulti".

2020 Grant for 1-year post-doc period abroad released by Sapienza University of Rome (Italy).

PUBLICATIONS (PEER REVIEWED JOURNALS)

1. **Giulia Federica Mancini**, Enrico Marchetta, Irene Pignani, Viviana Trezza, Patrizia Campolongo. Social defeat stress during early adolescence confers resilience against a single episode of prolonged stress in adult rats. *(Accepted for publication in Cells).*

2. **Giulia Federica Mancini**, Enrico Marchetta, Eleonora Riccardi, Viviana Trezza, Maria Morena, Patrizia Campolongo. Sex-divergent long-term effects of single prolonged stress in adult rats. Behav. Brain Res. 2020; 401:113096.

3. Maria Morena, Paola Colucci, **Giulia Federica Mancini**, Valentina De Castro, Andrea Peloso, Gustav Schelling, Patrizia Campolongo. Ketamine anesthesia enhances fear memory consolidation via noradrenergic activation in the basolateral amygdala. Neurobiol Learn Mem. 2020; 178:107362.

4. Paola Colucci, Enrico Marchetta, **Giulia Federica Mancini**, Phoebe Alva, Flavia Chiarotti, Mazahir T Hasan, Patrizia Campolongo. Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD). Transl Psychiatry. 2020; 10(1):243.

5. Paola Colucci[#], **Giulia Federica Mancini[#]**, Alessia Santori, Clemens Zwergel, Antonello Mai, Viviana Trezza, Benno Roozendaal, Patrizia Campolongo. Amphetamine and the smart drug 3,4-metmethylenedioxypyrovalerone (MDPV) induce generalization of fear memory in rats. Front Mol Neurosci. 2019; 12:292; #, equal contribution.

6. Alessia Santori, Paola Colucci, **Giulia Federica Mancini**, Maria Morena, Maura Palmery, Viviana Trezza, Stefano Puglisi-Allegra, Matthew N. Hill, Patrizia Campolongo. Anandamide modulation of circadian- and stress-dependent effects on rat short-term memory. Psychoneuroendocrinology. 2019; 108: 155-162.

Giulia Federica Mancini, Enrico Marchetta, Patrizia Campolongo. Long-term and sex-dependent effects of repeated brief social isolation stress during early adolescence on response to a second challenge in adult rats. *(In preparation)*.

PRESENTATIONS AT NATIONAL/INTERNATIONAL MEETINGS

1. **Mancini GF**, Marchetta E, Colucci Paola, Lucarella F, Campolongo P. Late glucocorticoid receptor antagonism normalizes the enduring effects induced by brief and repeated periods of social isolation stress during early- adolescence. Società Italiana

di Farmacologia (SIF) 40th Conference, 3-6 February 2021, Milano, Italy.

2. **Mancini GF**, Marchetta E, Splendori M, Filosa M, Campolongo P. Long-term effects of brief and repeated periods of social isolation stress during early adolescence. Società Italiana di Farmacologia (SIF) 39th Conference, 20-23 November 2019, Firenze, Italy.

3. Marchetta E, **Mancini GF**, Splendori M, Campolongo P. Social defeat stress during early adolescence affects susceptibility to stress-related disorders in adult rats. Società Italiana di Farmacologia (SIF) 39th Conference, 20-23 November 2019, Firenze, Italy.

4. **Mancini GF**, Marchetta E, Splendori M, Campolongo P. Enduring behavioral effects of brief and repeated periods of social isolation stress during early adolescence. 18th National Congress of the Italian Society for Neuroscience (SINS), 26-29 September 2019, Perugia, Italy.

5. Marchetta E, **Mancini GF**, Splendori M, Campolongo P. Social defeat stress during early adolescence affects susceptibility to stress-related disorders in adult rats. 18th National Congress of the Italian Society for Neuroscience (SINS), 26-29 September 2019, Perugia, Italy.

6. **Mancini GF**, Marchetta E, Splendori M, Campolongo P. Enduring effects induced by brief and repeated periods of social isolation stress during early adolescence. International Society of Psychoneuroendocrinology (ISPNE) Conference, 29-31 August 2019, Milano, Italy. Travel award winner.

7. **Mancini GF**, Marchetta E, Splendori M, Filosa M, Campolongo P. Long-term behavioral effects of brief and repeated periods of social isolation stress during early adolescence. Mediterranean Neuroscience Society 7th Conference 23- 27 June 2019, Marrakech, Morocco. Best poster prize winner.

8. Marchetta E, **Mancini GF**, Splendori M, Campolongo P. Social defeat stress during early adolescence affects susceptibility to stress-related disorders in adult rats. Mediterranean Neuroscience Society 7th Conference 23-27 June 2019, Marrakech, Morocco.

9. Colucci P; Marchetta E, **Mancini GF**, Marangio F, Puglisi Allegra S, Campolongo P. Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD). Mediterranean Neuroscience Society 7th Conference 23-27 June 2019, Marrakech, Morocco.

10. Santori A, Colucci P, **Mancini GF**, Ferrante P, Morena M, Hill MN,

Campolongo P. Endocannabinoid modulation of circadian- and stress- dependent effects on short-term memory in rats. Mediterranean Neuroscience Society 7th Conference 23-27 June 2019, Marrakech, Morocco.

11. **Mancini GF**, Colucci P, Splendori M, Roozendaal B, Campolongo P. The psychostimulants Amphetamine and the "bath salt" 3,4- methylenedioxypyrovalerone (MDPV) differentially affect the accuracy of memory in rats. 11th FENS Forum of Neuroscience (Federation of European Neuroscience Societies), 7-11 July 2018, Berlin, Germany.

12. Colucci P, **Mancini GF**, Santori A, Roozendaal B, Campolongo P. Amphetamine and the "bath salt" 3,4-methylenedioxypyrovalerone (MDPV) differentially affect the accuracy of memory for emotional experiences in rats. International Conference on Learning and Memory, 18-22 April 2018, UC Irvine, California (USA).

13. Colucci P, **Mancini GF**, Santori A, Roozendaal B, Campolongo P. Amphetamine and the 'bath salt' MDPV enhance generalization of memory for emotional experiences in rats. Mediterranean Neuroscience Society 6th Conference 12 – 15 June 2017, St Julian's Malta.

14. Colucci P, **Mancini GF**, Santori A, Campolongo P. Amphetamine and the "bath salt" 3,4-methylenedioxypyrovalerone (MDPV) alter accuracy of memory for emotional arousing experiences in rats. Società Italiana di Farmacologia (SIF) 38th Conference, 25-28 October 2017, Rimini, Italy.

APPENDIX

ANANDAMIDE MODULATION OF CIRCADIAN- AND STRESS-DEPENDENT EFFECTS ON RAT SHORT-TERM MEMORY

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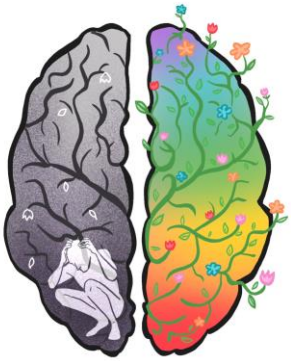
Psychoneuroendocrinology. 2019; 108:1055-162



Abstract

The endocannabinoid system plays a key role in the control of emotional responses to environmental challenges. CB1 receptors are highly expressed within cortico-limbic brain areas, where they modulate stress effects on memory processes. Glucocorticoid and endocannabinoid release is influenced by circadian rhythm. Here, we investigated how different stress intensities immediately after encoding influence rat short-term memory in an object recognition task, whether the effects depend on circadian rhythm and if exogenous augmentation of anandamide levels could restore any observed impairment. Two separate cohorts of male adult Sprague-Dawley rats were tested at two different times of the day, morning (inactivity phase) or afternoon (before the onset of the activity phase) in an object recognition task. The anandamide hydrolysis inhibitor URB597 was intraperitoneally administered immediately after the training trial. Rats were thereafter subjected to a forced swim stress under low or high stress conditions and tested 1-h after training. Control rats underwent the same experimental procedure except for the forced swim stress (no stress). We further investigated whether URB597 administration might modulate corticosterone release in rats subjected to the different stress conditions, both in the morning or afternoon. The low stressor elevated plasma corticosterone levels and impaired 1-h recognition memory performance when animals were tested in the morning. Exposure to the higher stress condition elevated plasma corticosterone levels and impaired memory performance, independently of the testing time. These findings show that stress impairing effects on short-term recognition memory are dependent on the intensity of stress and circadian rhythm. URB597 (0.3 mg kg^{-1}) rescued the altered memory performance and decreased corticosterone levels in all the impaired groups yet leaving memory unaltered in the non-impaired groups.

Introduction

The endocannabinoid system plays a key regulatory role in many fundamental physiological processes, such as sleep/wake cycles (Lovinger, 2008, Murillo-Rodríguez et al., 2017; Pava, et al., 2016), learning and memory (Akirav, 2011; Atsak et al., 2015; Morena and Campolongo, 2014) and central nervous system (CNS) regulation of endocrine functions (Hillard, 2015; Balsevich et al., 2017). The two major endocannabinoids, N-arachidonylethanolamide (anandamide, AEA; Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG; Sugiura et al., 1995) are synthesized on demand and travel retrogradely to presynaptic sites to bind cannabinoid type-1 (CB1) receptors (Kano et al., 2009). After being released into the synaptic cleft, AEA and 2-AG are primarily degraded by distinct hydrolytic enzymes, the fatty acid amide hydrolase (FAAH; Cravatt et al., 2001) and monoacylglycerol lipase (MAGL; Dinh et al., 2002), respectively.

Emotion influences memory at multiple levels (McGaugh, 2000), from perceptual recognition and identification (Zeelenberg et al., 2006) to explicit recognition and recall of emotional stimuli (Kensinger and Schacter, 2008). Compelling evidence indicates that drugs that target the endocannabinoid system induce biphasic effects on cognitive and emotional behavior depending on the level of stress and emotional arousal at the time of encoding and drug consumption (Campolongo et al., 2013; Manduca et al., 2014; Morena et al., 2014, 2015, 2016a). Glucocorticoids are stress response mediators which interact with the endocannabinoid system in the regulation of memory function (Campolongo et al., 2009; Hill et al., 2018; Morena and Campolongo, 2014). Their synthesis is characterized by a circadian release pattern, with peak levels linked to the start of the activity phase and diurnal regulation under control of the circadian clock (Dickmeis, 2009). Literature evidence indicates that the endocannabinoid signaling exhibits a circadian rhythm with variations reported in CB1 receptor expression (Rueda-Orozco et al., 2008), endocannabinoids tissue

contents and in the enzymes controlling their synthesis and degradation (Valenti et al., 2004). Extensive research has identified glucocorticoid-endocannabinoid crosstalk as crucial mediator of the glucocorticoid dependent modulation of emotional memories (Atsak et al., 2015; Campolongo et al., 2009), but still it remains uncertain the influence of circadian rhythm on this mediation. Moreover, far less well understood is the relationship between circadian rhythm biology and memory formation (Gerstner and Yin, 2010). Therefore, the main purpose of the present study was to evaluate how different stress intensities may influence short- term recognition memory in rats, investigating whether their action is regulated by circadian rhythm and if AEA has any role on this process. To this aim we investigated the effects of post-training systemic administration of the FAAH inhibitor, URB597, which increases AEA levels at active synapses, on short-term retention of object recognition memory under three different stress conditions (no, low or high forced swim stress), at two different times of the day, morning (inactivity phase) or afternoon (before the onset of the activity phase). Behavioral experiments were paralleled by biochemical measurement aimed at measuring plasma corticosterone levels in all the experimental groups.

Material and Methods

Animal Care and Use

Male adult Sprague-Dawley rats (350–450g at the time of training and testing, Charles River Laboratories, Calco, Italy) were kept individually in an air-conditioned colony room (temperature: 21 ± 1□C; lights on from 07:00 AM to 7:00 PM) with pellet food and water available ad libitum. Training and testing were performed during the light phase of the cycle between 10:00 AM and 6:00 PM. All procedures involving animal care or treatments were performed in compliance with

the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, and the D. L. 26/2014 of Italian Ministry of Health.

Drug Treatment

The anandamide hydrolysis inhibitor URB597 [(3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate] (0.1 or 0.3 mg kg-1; Tocris Bioscience, Bristol UK) was administered intraperitoneally (i.p.) in a volume of 1 ml kg-1 immediately after the training trial. Doses were chosen on the basis of pilot experiments performed in our laboratory and on literature data (Kathuria et al., 2003; Campolongo et al., 2013; Morena and Campolongo, 2014), in order to have a maximum augmentation of AEA release in the synaptic cleft. The solutions were freshly prepared on the day of the experiment and dissolved in 5% polyethylene glycol, 5% Tween-80 and 90% saline (vol/vol). The vehicle solution contained 5% polyethylene glycol and 5% Tween-80 in saline only.

Behavioral Procedures

Object recognition task. A slightly modified procedure of that described by Campolongo et al. (2013) was used. The experimental apparatus was a gray open-field box (in cm, 40 wide × 40 deep × 40 high) with the floor covered with sawdust, positioned in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). All rats were handled twice per day for 1 min each and extensively habituated to the experimental context twice per day for 3 min each for 7 days preceding the training day. During habituation, rats were allowed to freely explore the apparatus in the absence of objects. The animals were randomly assigned to three different groups: no stress, low stress and high stress conditions and tested either in the morning (rats' inactive phase, 10:00 AM - 12:30 PM) or in the afternoon (before the onset of the activity phase, 3:30 PM - 6:00 PM). On the

training trial, each rat was individually placed in the experimental apparatus at the opposite end from the objects. The rat was allowed to explore two identical objects (A1 and A2) for 6 min, then it was removed from the apparatus and, after drug treatment, if belonging to the low or high stress condition group, it was subjected to a forced swim stress; then, he was returned to his home cage. The no stress group was placed back to its home cage immediately after drug injection. To avoid the presence of olfactory trails, sawdust was stirred, foecal boli were removed and the objects were cleaned with 70% ethanol after each trial. Rat's behavior was recorded by using a video camera positioned above the experimental apparatus and videos were analyzed with Observer XT 12 (Noldus Information Technology BV, Wageningen, The Netherlands) by a trained observer who was unaware of treatment condition. Exploration of an object was defined as pointing the nose to the object at a distance of < 1 cm and/or touching it with the nose. Turning around or sitting on an object was not considered as exploration. During the training trial, the time spent exploring the two objects (total object exploration time, s) was taken as a measure of object exploration, and exploratory behavior of the experimental apparatus was analyzed by the measuring total number of crossings and rearings. For crossings, the floor of the apparatus was divided into four imaginary squares and the total number of crossings between squares was determined. Memory retention was tested 1 h after the training trial. On the retention test trial, one copy of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial (Fig. 1). All combinations and locations of objects were used to reduce potential biases due to preference for particular locations or objects. Each rat was placed in the apparatus for 6 min, and its behavior was recorded. To analyze cognitive performance, during the retention test, a discrimination index (DI) was calculated as the difference in time exploring the novel and the familiar object, expressed as the percentage ratio of the total time spent exploring both objects. Forced swim stress procedure. Forced swimming was used as the stressor because its neurochemical and

hormonal effects are well defined and meet the criteria of a stress-inducing agent (Schneider and Simson, 2007). Immediately after the training trial of the object recognition task rats were forced to swim in a tank (50 cm in height × 20 cm in diameter), filled to a depth of 30 cm with water. At the end of the swimming period, the rats were removed from the water and were immediately and gently wiped to dryness with absorbent paper before they were returned to the home cage. Rats in the low and high stress condition groups were subjected to a low or high intensity stressor by using a 1- or 5-min forced swim stress procedure at different water temperatures of 25 ± 1°C or 19 ± 1°C, respectively, known to elicit different plasma corticosterone levels (Morena et al., 2015).

Plasma Corticosterone Levels

Corticosterone levels were determined in rats in the no stress, low stress and high stress conditions that were tested in the morning or afternoon and in rats that were handled (twice per day for 7 days) but not trained (home cage), at the two different times of the day. As novelty stimulation triggers an HPA-axis response that leads to a corticosterone plasma peak at 30 min and normalizes within 90 min after stress exposure (de Kloet et al., 2005), rats were killed immediately after the test trial, 60 min after the URB597 administration. Trunk blood was collected after decapitation in tubes containing 200 µl of 0.1 M EDTA and samples were centrifuged at 1000 x g for 15 min at 4°C. Plasma was stored at –20°C and analyzed for corticosterone levels using a DetectX ELISA kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions as previously described (Fletcher et al., 2018). In compliance with EU animal legislation (3R principle: reduction) corticosterone levels were measured in vehicle-treated and in URB 0.3 mg/kg (effective dose in rescuing stress-dependent memory impairments) treated rats.

Data and Statistical Analysis

One-sample t-tests were used to determine whether the discrimination index was different from zero. Object recognition data and plasma corticosterone levels were analyzed by two-way ANOVAs. Tukey-Kramer post hoc tests were used to determine the source of the detected significances. P values of < 0.05 were considered statistically significant. To be included in the statistical analysis rats had to reach a minimum criterion of total object exploration time > 10 s on either training or testing. Prior findings indicate that such rats adequately acquire the task (Okuda et al., 2004; Roozendaal et al., 2008; Winters et al., 2009; Campolongo et al., 2013; Barsegyan et al., 2019). All data are expressed as mean ± standard error of the mean (SEM).

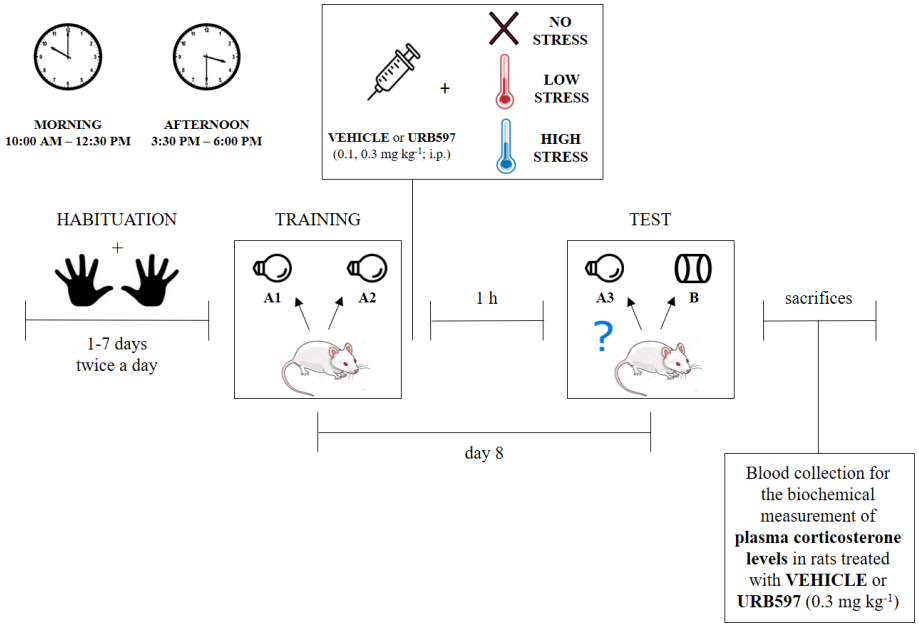


Figure 1 – Diagram of the experimental procedure.

Results

Effects of different stress intensities and circadian rhythm on short-term recognition memory retention performance and plasma corticosterone levels

To determine whether different stress intensities modulate short-term memory retention performance and whether these effects are dependent on circadian rhythm, we first analyzed the behavioral performance of all vehicle-treated rats, used in the subsequent URB597 experiments, at different times of the day (e.g. morning and afternoon), in order to unveil any possible influence of stress or time on memory and corticosterone levels.

One-sample t-tests revealed that the discrimination indexes of vehicle-treated rats were significantly different from zero for both the no stress groups tested either in the morning or in the afternoon ($t_{(7)} = 4.654$, $P = 0.002$ and $t_{(9)} = 4.384$, $P = 0.002$, respectively; Fig. 2a) and for the low stress condition group tested in the afternoon ($t_{(10)} = 3.715$, $P = 0.004$; Fig. 2a), thus indicating that these three animal groups discriminated the novel object. In contrast, rats in the remaining low and high stress conditions morning groups and the high stress condition afternoon group did not express memory retention for the familiar object. Two-way ANOVA for discrimination index revealed a significant stress condition effect ($F_{(2.50)} = 4.313$, $P = 0.019$) and a tendency toward significance for the time of the testing ($F_{(1.50)} = 3.082$, $P = 0.085$) and for the interaction between these two factors ($F_{(2.50)} = 2.493$, $P = 0.093$). *Post hoc* analysis showed that the low stress condition significantly decreased the discrimination index of rats tested in the morning as compared to the no stress group tested at the same time of the day and the corresponding low stress condition group tested in the afternoon ($P < 0.05$ for both comparisons; Fig. 2a).

Regarding the total object exploration time on the testing trial, two-way ANOVA revealed a significant stress condition effect ($F_{(2.50)} = 12.693$, $P < 0.0001$), but no significant time of testing or stress condition x time of testing interaction effects. Finally, rats' exploratory behavior of the apparatus during the test trial did not differ

among the different experimental groups. Two-way ANOVAs for number of crossings or rearings revealed no significant stress condition, no time of testing or stress condition x time of testing interaction effects (Table 1).

Furthermore, we evaluated whether plasma corticosterone levels were differentially modulated by the different stress conditions, at two times of the day.

Two-way ANOVA for plasma corticosterone levels immediately after test, revealed a significant stress condition effect ($F_{(3.54)} = 17.836$, $P < 0.0001$), but no significant time or stress condition x time effects. *Post hoc* analysis showed that rats that were subjected to low stress condition had higher corticosterone levels than home cage control rats only in the morning ($P < 0.01$; Fig. 2b). Moreover, rats subjected to the high stress condition presented significant higher corticosterone levels than home cage control rats and no stress groups both in the morning ($P < 0.01$, for both comparisons; Fig. 2b) and in the afternoon ($P < 0.01$ and $P < 0.05$; for home cage and no stress groups, respectively; Fig. 2b).

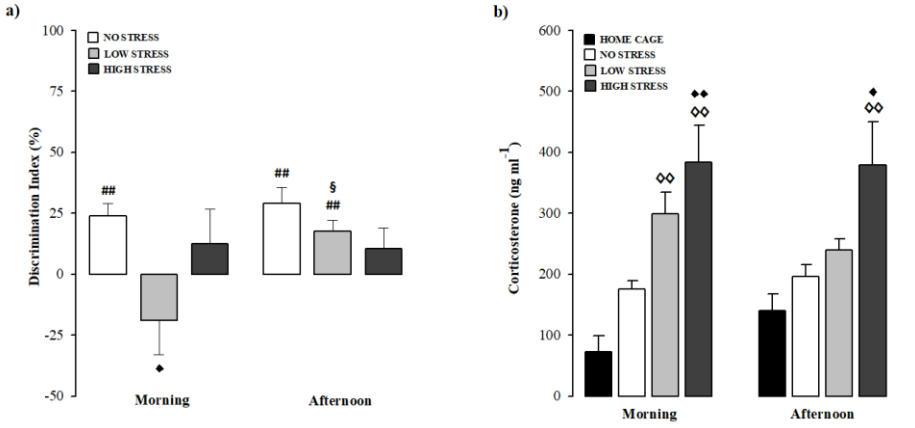


Figure 2 – Circadian-dependent effects of different stress conditions on short-term memory. **a)** Discrimination index on the testing trial for vehicle-treated rats that were subjected to no, low or high stress conditions immediately after the training trial performed in

the morning or afternoon. *Post hoc* comparisons reported significant differences between groups as follows: ♦ $P < 0.05$ vs the corresponding no stress group. § $P < 0.05$ vs the corresponding low stress group trained in the morning. ## $P < 0.01$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 8-11 per group). **b)** Plasma corticosterone levels of home cage and vehicle-treated rats subjected to no, low or high stress condition immediately after the training trial that were euthanized, in the morning or in the afternoon, 60 min after stress exposure, immediately after test. *Post hoc* comparisons reported significant differences between groups as follows: ◊◊ $P < 0.01$ vs the corresponding home cage group. ♦ $P < 0.05$; ♦♦ $P < 0.01$ vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 7-9 per group).

Table 1
Exploratory behavior on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial, in the morning and in the afternoon sessions.

	Morning			Afternoon		
	Total object exploration time (s)	Number of crossings	Number of rearings	Total object exploration time (s)	Number of crossings	Number of rearings
NO STRESS						
VEHICLE	58.2 ± 8.2	21.4 ± 4.2	26.4 ± 5.8	46.3 ± 7.5	17.6 ± 4.1	26.6 ± 4.8
URB 0.1	50.6 ± 9.4	20.8 ± 4.1	34.6 ± 6.7	68.5 ± 18.3	24.0 ± 4.9	31.1 ± 5.4
URB 0.3	47.0 ± 5.7	23.6 ± 3.6	30.5 ± 4.9	48.4 ± 7.0	20.0 ± 4.0	26.4 ± 4.4
LOW STRESS						
VEHICLE	33.4 ± 5.9 *	23.0 ± 3.7	39.1 ± 4.6	32.8 ± 4.0	16.9 ± 2.3	28.7 ± 3.7
URB 0.1	32.4 ± 6.4	16.8 ± 2.1	32.0 ± 4.3	31.5 ± 5.7 *	13.8 ± 3.1	24.6 ± 4.3
URB 0.3	20.9 ± 3.9 **	16.9 ± 2.3	31.8 ± 5.8	35.6 ± 5.0	19.8 ± 2.5	26.1 ± 3.8
HIGH STRESS						
VEHICLE	17.0 ± 4.8 **	15.6 ± 3.4	43.0 ± 6.8	30.9 ± 3.0	15.9 ± 2.0	34.6 ± 8.6
URB 0.1	17.3 ± 4.0 *	13.6 ± 3.5	33.6 ± 9.0	29.9 ± 3.4 *	10.9 ± 1.1	39.1 ± 4.7
URB 0.3	15.5 ± 3.6 **	17.3 ± 2.5	37.8 ± 4.0	28.1 ± 4.8 *	10.5 ± 1.3	30.0 ± 7.6

Total time spent exploring the two objects (in seconds) and the number of crossings and rearings of all groups tested in the morning and in the afternoon. * $P < 0.05$; ** $P < 0.01$ vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 8–12 per group).

Effects of the AEA hydrolysis inhibitor URB597 on short-term object recognition memory performance and plasma corticosterone levels in the no, low and high stress condition groups tested in the morning

This experiment investigated whether immediate post-training injection of the AEA hydrolysis inhibitor URB597 modulates short-term performance on an object recognition task and plasma corticosterone levels and whether these effects are influenced by different stress conditions in animals tested in the morning.

As shown in figure 3a, one-sample t-tests revealed that the discrimination indexes were significantly different from zero for all no stress treatment groups ($t_{(7)} = 4.654$, $P = 0.002$; $t_{(7)} = 2.741$, $P = 0.029$ and $t_{(7)} = 4.745$, $P = 0.002$; vehicle, URB597 0.1

and URB597 0.3 mg kg^{-1}, respectively), while, for the low and high stress groups, only URB597 0.3 mg kg^{-1}treated rats discriminated the new object ($t_{(7)}$ = 3.206, P = 0.015, $t_{(7)}$ = 5.533, P = 0.001, for the low and high stress conditions URB597 0.3 mg kg^{-1}groups, respectively). In contrast, low and high stressed rats in the remaining vehicle and URB597 0.1 mg kg^{-1} groups did not express memory retention for the familiar object. Two-way ANOVA for the discrimination index revealed significant stress condition ($F_{(2.63)}$ = 3.838, P = 0.027) and treatment ($F_{(2.63)}$ = 7.257, P = 0.002) effects as well as a tendency toward significance for the interaction between these two factors ($F_{(4.63)}$ = 2.112, P = 0.090). *Post hoc* analysis showed that URB597 0.3 mg kg^{-1} treated rats subjected to low or high stress presented a better discrimination index relative to their corresponding vehicle groups (P < 0.05, for both comparisons; Fig. 3a). Moreover, rats that were treated with URB597 0.3 mg $kg^{-1}$and then subjected to the high stress condition showed a high discrimination index as compared to those administered the same dose of URB597 but subjected to the no or low stress procedure (P < 0.05, for both comparisons; Fig. 3a). Concerning the total exploration time of the two objects on the testing trial, two-way ANOVA revealed a significant stress condition effect ($F_{(2.63)}$ = 24.885, P < 0.0001), but no significant treatment or stress condition x treatment effects. Finally, rats' exploratory behavior of the apparatus during the test trial did not differ among the different experimental groups. Two-way ANOVAs for number of crossings and rearings revealed no significant stress condition, treatment or stress condition x treatment interaction effects (Table 1).

Two-way ANOVA for plasma corticosterone levels revealed significant stress condition ($F_{(2.41)}$ = 6.969, P = 0.003) and treatment ($F_{(1.41)}$ = 10.634, P = 0.002) effects, but no significant interaction between these two factors. *Post hoc* analysis showed that URB597 0.3 mg kg^{-1} treated rats subjected to low or high stress presented lower corticosterone levels than their corresponding vehicle groups (P < 0.05, for both comparisons; Fig. 3b), suggesting that URB597 0.3 mg kg^{-1}

counteracted the stress-induced increase on plasma corticosterone levels, in both the low and high stress conditions.

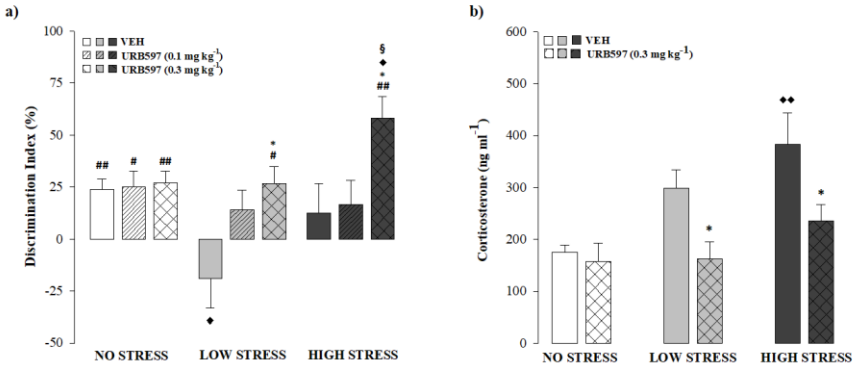


Figure 3 – URB597 modulation of stress-dependent effects on short-term memory in the morning. a) Discrimination index on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial performed in the morning. *Post hoc* comparisons reported significant differences between groups as follows: * $P < 0.05$ vs the corresponding vehicle group. ♦ $P < 0.05$ vs the corresponding no stress group. § $P < 0.05$ vs the corresponding low stress group. # $P < 0.05$; ## $P < 0.01$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 8-9 per group). **b)** Plasma corticosterone levels of vehicle and URB597 0.3 mg kg^{-1} treated rats subjected to no, low or high stress condition immediately after the training trial that were euthanized in the morning, 60 min after stress exposure, immediately after test. *Post hoc* comparisons reported significant differences between groups as follows: * $P < 0.05$ vs the corresponding vehicle group. ♦♦ $P < 0.01$ vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 6-9 per group).

Effects of the AEA hydrolysis inhibitor URB597 on short-term object recognition memory performance and plasma corticosterone levels in the no, low and high stress condition groups tested in the afternoon

This experiment investigated whether immediate post-training injection of the AEA

hydrolysis inhibitor URB597 altered short-term performance on an object recognition task and plasma corticosterone levels and whether these effects were influenced by different stress conditions (no, low and high stress) when animals were tested in the afternoon.

As shown in figure 4a, one-sample t-tests revealed that the discrimination indexes were significantly different from zero for the no stress and low stress vehicle, URB597 0.1 mg kg^{-1} and URB597 0.3 mg kg^{-1} groups ($t_{(9)} = 4.384$, $P = 0.002$; $t_{(8)} = 2.658$, $P = 0.029$ and $t_{(7)} = 2.805$, $P = 0.026$, respectively for no stress groups; $t_{(10)} = 3.715$, $P = 0.004$; $t_{(10)} = 2.435$, $P = 0.035$ and $t_{(10)} = 4.412$, $P = 0.001$, respectively for low stress condition groups) and the high stress condition URB597 (0.1 and 0.3 mg kg^{-1}) groups ($t_{(11)} = 3.266$, $P = 0.008$; $t_{(11)} = 7.987$, $P < 0.0001$), thus indicating that these animals discriminated the novel object with respect to the familiar one. Rats in the remaining high stress vehicle group did not express memory retention for the familiar object (Fig. 4a). Two-way ANOVA for discrimination index revealed no significant stress condition or treatment effects, but a significant interaction between these two factors ($F_{(4.86)} = 2.593$, $P = 0.042$). *Post hoc* comparisons showed that, among rats tested under the high stress condition, URB597 0.3 mg kg^{-1} significantly increased the discrimination index as compared to vehicle treated rats ($P < 0.01$; Fig. 4a). Moreover, rats treated with the high dose of URB597 and subjected to the high stress condition presented a significant high discrimination index as compared to their corresponding low stress group ($P < 0.05$; Fig. 4a). Concerning the total exploration time of the two objects on the testing trial, two-way ANOVA revealed a significant stress condition effect ($F_{(2.86)} = 9.794$, $P = 0.0001$), but no significant treatment or stress condition x treatment effect (Table 1). Two-way ANOVA for number of crossings revealed a significant stress condition effect ($F_{(2.86)} = 5.902$, P

= 0.004), but no significant treatment or stress condition x treatment interaction effects (Table 1). Concerning the number of rearings, two-way ANOVA revealed no significant stress condition effect, no treatment effect or any interaction between these two factors (Table 1).

Two-way ANOVA for plasma corticosterone levels revealed significant treatment ($F_{(1.37)} = 6.169$, $P = 0.018$) and stress condition x treatment interaction ($F_{(2.37)} = 6.289$, $P = 0.005$) effects, but no significant effect of the stress condition. *Post hoc* analysis showed that only URB597 0.3 mg kg^{-1} treated rats subjected to high stress presented lower corticosterone levels than their corresponding vehicle group ($P < 0.01$; Fig. 4b), suggesting that URB597 0.3 mg kg^{-1} counteracted the stress-induced increase on plasma corticosterone levels in the high stress condition.

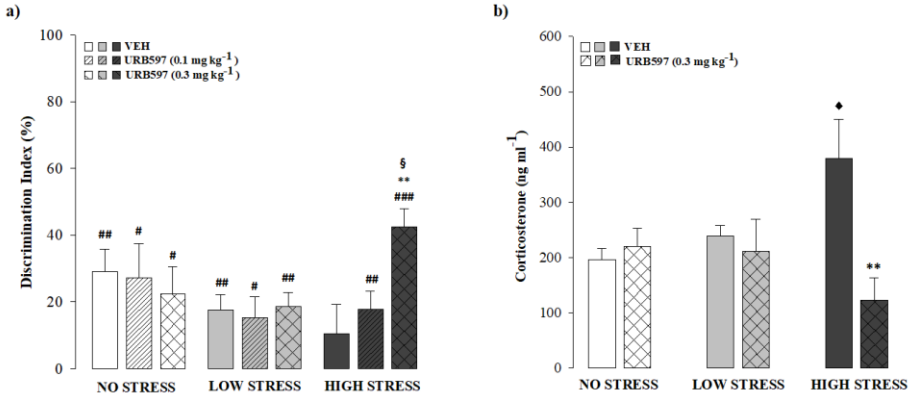


Figure 4 – URB597 modulation of stress-dependent effects on short-term memory in the afternoon. a) Discrimination index on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial, in the afternoon session. *Post hoc* comparisons reported significant differences between groups as follows: ** $P < 0.01$ vs the corresponding vehicle group. § $P < 0.05$ vs the corresponding low stress group. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.0001$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 8-12 per group). **b)** Plasma corticosterone levels of vehicle and URB597 0.3 mg kg^{-1} treated rats subjected to no, low or

high stress condition immediately after the training trial that were euthanized in the afternoon, 60 min after stress exposure, immediately after test. *Post hoc* comparisons reported significant differences between groups as follows: ** $P < 0.01$ vs the corresponding vehicle group. ♦ $P < 0.05$ vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 6-8 per group).

Discussion

The present findings show that systemic administration of the AEA hydrolysis inhibitor URB597 counteracts the stress impairing effects on short-term object recognition memory, in a stress intensity- and circadian-dependent fashion. We have previously shown that activation of CB1 receptors differentially modulates short-term recognition memory in rats depending on environmental aversiveness and on the level of stress the animal experienced at the time of drug administration and memory encoding (Campolongo et al., 2013, 2012). In particular, post-training administration of the CB1 receptor agonist WIN55,212-2 enhanced object recognition performance (tested 24 hour later) exclusively in animals training under a high arousal state (Campolongo et al., 2013). Literature data suggested that low versus high doses of THC and synthetic cannabinoid agonists provoke opposite stress-induced corticosterone release through CB1-mediated mechanisms (Mayer et al., 2014; Patel et al., 2004; Sano et al., 2009). Evidence has indicated that endocannabinoid augmentation approaches via FAAH or MAGL inhibitors generally produce dose-related decreases in the regulation of HPA-axis function and anxiety, whereas THC and exogenous cannabinoids produce biphasic effects with low doses mimicking endocannabinoid augmentation effects (Hill et al., 2018). Although there is one report showing that systemic administration of the FAAH inhibitor URB597 impairs the acquisition and early consolidation of contextual fear conditioning (Burman et al., 2016), other studies investigating the AEA signaling indicated that URB597 treatment enhanced consolidation (Morena et al., 2014) and impaired retrieval of aversive memories throughout indirect CB1 activation (Ratano

et al., 2014). CB1 receptors are abundantly expressed in cortico-limbic regions, including the basolateral complex of the amygdala (BLA), hippocampus and medial prefrontal cortex (mPFC), where they modulate emotional arousal effects on memory (Akirav, 2013; Morena et al., 2015, 2014; Tasker et al., 2015) and regulate hypothalamic–pituitary–adrenal (HPA) axis activity (Morena et al., 2016b). Extensive research has demonstrated that not only CB1 receptors, but also glucocorticoid receptors are located within this brain circuitry (Herkenham et al., 1990; Hill et al., 2010; Myers et al., 2014). Numerous evidence shows that glucocorticoids enhance memory consolidation of emotionally arousing experiences, but impair memory retrieval and working memory (de Quervain et al., 2017; McIntyre and Roozendaal, 2007). These different glucocorticoids effects are dependent on a non-genomically mediated interaction with noradrenergic transmission within the BLA and the hippocampus, wherein the endocannabinoid system has been shown to play an important role in mediating such effects (Atsak et al., 2015, 2012a; Jiang et al., 2014). Specifically, glucocorticoids or a stressor, administered shortly before or immediately after training, impair short-term memory performances in an object recognition task (Okuda et al., 2004; Roozendaal et al., 2006b), likely by negatively interfering with memory retrieval. Similarly, intrahippocampal infusions of the cannabinoid agonist WIN55,212–2 impair the retrieval of memory (Morena et al., 2015); however, antagonism of hippocampal β-adrenoceptor activity blocks the memory retrieval impairment induced by WIN55,212–2 (Atsak et al., 2012a), supporting the evidence that glucocorticoid and endocannabinoid signaling interact to impair the retrieval of emotional memory through their influence on downstream noradrenergic activity (Balsevich et al., 2017). The locus coeruleus (LC), the main source of norepinephrine in the mammalian forebrain, provides norepinephrine to different brain regions, including the BLA (McCall et al., 2017) and mPFC (Sara, 2009), wherein activation of CB1

receptors results in decreased cortical norepinephrine release (Reyes et al., 2012), when it is normally potentiated by acute swim stress exposure (Morilak et al., 2005). Evidence suggests that under high levels of stress the LC promotes fear learning by enhancing BLA function, while simultaneously blunting prefrontal function. Conversely, low levels of arousal are sufficient for the LC to facilitate mPFC function and promote downstream inhibition of the amygdala (Giustino and Maren, 2018). Herein we demonstrated that exposure to a low stress immediately after the training trial selectively impairs short-term memory retention/retrieval when animals are tested in the morning while exposure to a high stress impairs short-term performance independently of the testing time. Interestingly, the stressed groups that were unable to discriminate between the 2 objects were those presenting increased levels of corticosterone. This is in accordance with extensive human and animal research showing that glucocorticoids impair memory retrieval (Roozendaal et al., 2006a; Wolf et al., 2016; de Quervain et al., 2019). Interestingly, our findings showed that post-training treatment with the AEA hydrolysis inhibitor URB597 counteracts these impairing effects of stress on memory performance, both in the morning and afternoon testing sessions. Specifically, systemic URB597 injection, at the dose of 0.3 mg kg^{-1}, enhances short-term memory retention in the low stress condition group tested in the morning, as well as in both the high stress groups tested either in the morning or in the afternoon, maintaining unaltered the performances of rats that did not show any cognitive impairment. Extensive evidence indicates that cannabinoids, either administered exogenously or released from endogenous sites, have pronounced effects on learning and memory (Hill et al., 2018; Marsicano and Lafenêtre, 2009; Morena and Campolongo, 2014; Ratano et al., 2017). Moreover, previous evidence has shown that AEA and 2-AG modulate emotional memory processes by interacting with glucocorticoids and other stress-activated neuromodulatory systems such as norepinephrine, in brain limbic regions (Atsak et al., 2015, 2012b; Campolongo et al., 2009; Morena et al., 2016a, 2015, 2014;

Morena and Campolongo, 2014). Our finding that URB597 treatment has no effects in animals tested under no stress condition but selectively affects memory in the presence of a stressor, is in line with this evidence and has a high impact potential. On the light of this evidence it is tentative to speculate that stress of different intensities at two times of the day differentially regulated LC-NE action on the mPFC, since such interaction might be described by an inverted-U function such that it can either enhance or hinder learning depending on different arousal states (Giustino and Maren, 2018). The exact mechanisms underlying cannabinoid modulation of norepinephrine has yet to be determined, but evidence indicated that it may involve direct influences of CB1 receptors that are localized to noradrenergic axon terminals in the mPFC (Oropeza et al., 2007), which contribute to regulating norepinephrine release. In particular, microdialysis data supported a mechanism whereby administration of WIN55,212–2 prior to swim stress exposure decreased cortical norepinephrine efflux by inhibiting presynaptic inhibitory α2-adrenergic autoreceptors (Reyes et al., 2012), and such evidence is supported by predominant presynaptic distribution of α2-adrenergic receptors in the mPFC (Cerrito and Preziosi, 1985; Dennis et al., 1987; Pudovkina et al., 2001). It is well known that stress effects on memory performance follow an inverted U- shaped relationship; very low or very high levels of stress have detrimental effects, while intermediate levels lead to optimal memory performances (Baldi and Bucherelli, 2005). In mammals, an important feature of glucocorticoid regulation is a diurnal release pattern, with serum cortisol/corticosterone concentration peak in the morning and lowest at night (Dickmeis, 2009). Since rats are nocturnal animals, under laboratory circumstances of a regular light/dark cycle, the peak of HPA rhythm occurs in the afternoon, just before the onset of the activity phase; the nadir occurs during sleep, when corticosterone levels reach their lowest serum concentration, whereas in the morning (during the rats' inactive phase) the HPA axis activity begins to increase (Bertani et al., 2010; Gong et al., 2015). Although different studies have

demonstrated that circadian clocks can influence learning and different studies have demonstrated that circadian clocks can influence learning and memory function (Tapp and Holloway, 1981; Gerstner and Yin, 2010; Smarr et al., 2014), no circadian effect has been documented on short-term memory recognition performances yet. Our results show that vehicle-treated animals tested in the morning session have impaired memory retention when exposed to both low or high stressors. These groups of rats also presented higher plasma corticosterone levels than no stress group. However, when vehicle-treated rats were tested in the afternoon, memory retention was only negatively affected by the exposure to the high stressor, which in parallel increased rats' plasma corticosterone levels. It is tentative to speculate that when animals are tested during the low activity phase of the HPA axis (i.e. morning session), both low and high stressor exposures induce a severe deviation from homeostasis which negatively affects memory retention performance. Our finding that exposure to low and high stress conditions elevated plasma corticosterone levels in rats that were trained in the morning, is in line with this evidence. Conversely, when animals are tested at the beginning of their active phase (i.e. afternoon), at their plasma corticosterone concentration peak, the high, but not the low, stress exposure might induce a more robust deflection from homeostasis, thus only the high stress condition group presents impairments in memory retention performance and higher plasma corticosterone levels. Our results indicate that maximal memory strength requires an intermediate level of stress, thus are in line with the Yerkes-Dodson law. Of note, boosting AEA levels with systemic URB597 injections is capable to specifically counteract these stress detrimental effects on short-term memory performance, decreasing plasma corticosterone levels in impaired memory groups. Previous findings indicated that WIN55,212-2 inhibited stress-induced elevation in corticosterone levels (Campolongo et al., 2013; Ganon- Elazar and Akirav, 2012, 2009), ameliorating the detrimental effects of stress on memory. Nevertheless, evidence demonstrated that the effects of cannabinoid drugs such as WIN55,212-2 on plasma corticosterone levels strictly depend on the level of arousal at the moment

of administration. Previous findings demonstrated that URB597 is capable to reduce plasma corticosterone levels in response to repeated stress exposures (Hill et al., 2010). Whether this URB597 effect is due to an interaction with the HPA axis activity or to a direct effect on memory performance, or both, needs to be further investigated, but the current data strongly indicate that URB597 is able to reduce plasma corticosterone levels in short-term memory impaired-groups. Taken together, our findings indicate that stress impairing effects on short-term recognition memory seem to be dependent on the intensity of stress and HPA axis circadian rhythm and that treatment with URB597 is capable of specifically counteracting these detrimental effects. These results suggest that FAAH inhibition may be a potential therapeutic target for stress-inducing memory alterations highlighting the need for clinical studies to examine this possible cannabinoid mechanism of restoring memory impairments.

References

Akirav I (2013) Cannabinoids and glucocorticoids modulate emotional memory after stress. Neurosci. Biobehav. Rev. 37, 2554–2563.

Akirav I (2011) The role of cannabinoids in modulating emotional and non-emotional memory processes in the hippocampus. Front. Behav. Neurosci. 5, 34.

Antunes M, Biala G (2012) The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn. Process. 13, 93–110.

Atsak P, Hauer D, Campolongo P, Schelling G, Fornari RV, Roozendaal B (2015) Endocannabinoid Signaling within the Basolateral Amygdala Integrates Multiple Stress Hormone Effects on Memory Consolidation. Neuropsychopharmacology 40, 1485–1494.

Atsak P, Hauer D, Campolongo P, Schelling G, McGaugh JL, Roozendaal B (2012a) Glucocorticoids interact with the hippocampal endocannabinoid system in impairing retrieval of contextual fear memory. Proc. Natl. Acad. Sci. U. S. A. 109, 3504–3509.

Atsak P, Roozendaal B, Campolongo P (2012b) Role of the endocannabinoid system in regulating glucocorticoid effects on memory for emotional experiences. Neuroscience 204, 104–116.

Baldi E, Bucherelli C (2005) The inverted “U-Shaped” Dose-Effect Relationships in Learning

and Memory: Modulation of Arousal and Consolidation. Nonlinearity Biol. Toxicol. Balsevich G, Petrie GN, Hill MN (2017) Endocannabinoids: Effectors of glucocorticoid signaling. Front. Neuroendocrinol. 47, 86–108.

Barsegyan A, Mirone G, Ronzoli G, Guo C, Song Q, van Kuppeveld D, Schut EHS, Atsak P, Teurlings S, McGaugh JL, Schubert D, Roozendaal B (2019) Glucocorticoid enhancement of recognition memory via basolateral amygdala-driven facilitation of prelimbic cortex interactions. Proc. Natl. Acad. Sci. U. S. A. 116, 7077-7082.

Bertani S, Carboni L, Criado A, Michielin F, Mangiarini L, Vicentini E (2010) Circadian profile of peripheral hormone levels in Sprague-Dawley rats and in common marmosets (Callithrix jacchus). In Vivo (Brooklyn). 24, 827–836.

Burman MA, Szolusha K, Bind R, Kerney K, Boger DL, Bilsky EJ (2016) FAAH inhibitor OL-135 disrupts contextual, but not auditory, fear conditioning in rats. Behav. Brain Res. 308, 1–5.

Campolongo P, Morena M, Scaccianoce S, Trezza V, Chiarotti F, Schelling G, Cuomo V, Roozendaal B (2013) Novelty-induced emotional arousal modulates cannabinoid effects on recognition memory and adrenocortical activity. Neuropsychopharmacology 38, 1276–86.

Campolongo P, Ratano P, Manduca A, Scattoni ML, Palmery M, Trezza V, Cuomo V (2012) The endocannabinoid transport inhibitor AM404 differentially modulates recognition memory in rats depending on environmental aversiveness. Front. Behav. Neurosci. 6, 11.

Campolongo P, Roozendaal B, Trezza V, Hauer D, Schelling G, McGaugh JL, Cuomo V (2009) Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. Proc. Natl. Acad. Sci. U. S. A. 106, 4888–4893.

Cerrito F, Preziosi P (1985) Rat brain alpha 2-pre- and postsynaptic receptors are different or differently modulated? J Neurosci Res. 14, 423-431.

Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc. Natl. Acad. Sci. U. S. A. 98, 9371–9376.

Davies JA, Navaratnam V, Redfern PH (1973) A 24-hour rhythm in passive-avoidance behaviour in rats. Psychopharmacologia 32, 211–214.

de Kloet ER, Joëls M, Holsboer (2005) Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6, 463–475.

de Quervain DJ, Schwabe L, Roozendaal B (2017) Stress, glucocorticoids and memory: implications for treating fear-related disorders. Nat. Rev. Neurosci. 18, 7–19.

de Quervain DJ, Wolf OT, Roozendaal B (2019) Glucocorticoid-induced enhancement of extinction-from animal models to clinical trials. Psychopharmacology (Berl) 236, 183-199.

Dennis T, L'Heureux R, Carter C, Scatton B (1987) Presynaptic alpha-2 adrenoceptors play a

major role in the effects of idazoxan on cortical noradrenaline release (as measured by in vivo dialysis) in the rat. J Pharmacol Exp Ther. 241, 642-649.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–9.

Dickmeis T (2009) Glucocorticoids and the circadian clock. J. Endocrinol. 200, 3–22Med. 3, 9–21.

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc. Natl. Acad. Sci. U. S. A. 99, 10819–24.

Donley MP, Schulkin J, Rosen JB (2005) Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual fear. Behav. Brain Res. 164, 197–205.

Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2005) Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. J. Pharmacol. Exp. Ther. 313, 352–8.

Fletcher K, Xiong Y, Fletcher E, Gustafsson L (2018) Glucocorticoid response to both predictable and unpredictable challenges detected as corticosterone metabolites in collared flycatcher droppings. PLoS One 13, e0209289.

Ganon-Elazar E, Akirav I (2012) Cannabinoids Prevent the Development of Behavioral and Endocrine Alterations in a Rat Model of Intense Stress. Neuropsychopharmacology 37, 456–466.

Ganon-Elazar E, Akirav I (2009) Cannabinoid Receptor Activation in the Basolateral Amygdala Blocks the Effects of Stress on the Conditioning and Extinction of Inhibitory Avoidance. J. Neurosci. 29, 11078–11088.

Gerstner JR, Yin JCP (2010) Circadian rhythms and memory formation. Nat. Rev. Neurosci. 11, 577–88.

Giustino TF, Maren S (2018) Noradrenergic Modulation of Fear Conditioning and Extinction. Front Behav Neurosci. 12, 43.

Gong S, Miao Y-L, Jiao G-Z, Sun M-J, Li H, Lin J, Luo M-J, Tan J-H (2015) Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. PLoS One 10, e0117503.

Haller J, Barna I, Barsvari B, Gyimesi Pelczer K, Yasar S, Panlilio LV, Goldberg S (2009) Interactions between environmental aversiveness and the anxiolytic effects of enhanced cannabinoid signaling by FAAH inhibition in rats. Psychopharmacology (Berl). 204, 607–616.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC (1990) Cannabinoid receptor localization in brain. Proc. Natl. Acad. Sci. U. S. A. 87, 1932–6.

Hill MN, Campolongo P, Yehuda R, Patel S (2018) Integrating Endocannabinoid Signaling

and Cannabinoids into the Biology and Treatment of Posttraumatic Stress Disorder. Neuropsychopharmacology 43, 80–102.

Hill MN, McLaughlin RJ, Bingham B, Shrestha L, Lee TTY, Gray JM, Hillard CJ, Gorzalka BB, Viau V (2010) Endogenous cannabinoid signaling is essential for stress adaptation. Proc. Natl. Acad. Sci. U. S. A. 107, 9406–9411.

Hillard CJ (2015) Endocannabinoids and the Endocrine System in Health and Disease, in: Handbook of Experimental Pharmacology. pp. 317–339.

Holloway FA, Wansley RA (1973) Multiple retention deficits at periodic intervals after active and passive avoidance learning. Behav. Biol. 9, 1–14.

Horn H, Böhme B, Dietrich L, Koch M (2018) Endocannabinoids in Body Weight Control. Pharmaceuticals 11, 55.

Jiang C-L, Liu L, Tasker JG (2014) Why do we need nongenomic glucocorticoid mechanisms? Front. Neuroendocrinol. 35, 72–5.

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009) Endocannabinoid-Mediated Control of Synaptic Transmission. Physiol. Rev. 89, 309–380.

Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, Rana G, La Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat. Med. 9, 76–81.

Kensinger EA, Schacter DL (2008) Neural Processes Supporting Young and Older Adults' Emotional Memories. J. Cogn. Neurosci. 20, 1161–1173.

Lovinger DM (2008) Regulation of Synaptic Function by Endocannabinoids. Learn. Mem. A Compr. Ref. 771–792.

Manduca A, Servadio M, Campolongo P, Palmery M, Trabace L, Vanderschuren LJMJ, Cuomo V, Trezza V (2014) Strain- and context-dependent effects of the anandamide hydrolysis inhibitor URB597 on social behavior in rats. Eur. Neuropsychopharmacol. 24, 1337–48.

Marsicano G, Lafenêtre P (2009) Roles of the endocannabinoid system in learning and memory. Curr. Top. Behav. Neurosci. 1, 201–30.

Mayer TA, Matar MA, Kaplan Z, Zohar J, Cohen H (2014) Blunting of the HPA-axis underlies the lack of preventive efficacy of early post-stressor single-dose Delta-9-tetrahydrocannabinol (THC). Pharmacol. Biochem. Behav. 122, 307–318.

McCall JG, Siuda ER, Bhatti DL, Lawson LA, McElligott ZA, Stuber GD, Bruchas MR (2017) Locus coeruleus to basolateral amygdala noradrenergic projections promote anxiety-like behavior. Elife 6.

McGaugh JL (2000) Memory--a century of consolidation. Science 287, 248–51.

McIntyre CK, Roozendaal B (2007) Adrenal Stress Hormones and Enhanced Memory for Emotionally Arousing Experiences, Neural Plasticity and Memory: From Genes to Brain Imaging.

Morena M, Campolongo P (2014) The endocannabinoid system: An emotional buffer in the

modulation of memory function. Neurobiol. Learn. Mem. 112, 30–43.

Morena M, De Castro V, Gray JM, Palmery M, Trezza V, Roozendaal B, Hill MN, Campolongo P (2015) Training-Associated Emotional Arousal Shapes Endocannabinoid Modulation of Spatial Memory Retrieval in Rats. J. Neurosci. 35, 13962–13974.

Morena M, Leitl KD, Vecchiarelli HA, Gray JM, Campolongo P, Hill MN (2016a) Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety. Neuropharmacology 111, S397–S402.

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

Morena M, Roozendaal B, Trezza V, Ratano P, Peloso A, Hauer D, Atsak P, Trabace L, Cuomo V, McGaugh JL, Schelling G, Campolongo P (2014) Endogenous cannabinoid release within prefrontal-limbic pathways affects memory consolidation of emotional training. Proc. Natl. Acad. Sci. U. S. A. 111, 18333–8.

Morilak DA (2012) Modulating the modulators: Interaction of brain norepinephrine and cannabinoids in stress. Exp. Neurol. 238, 145–148.

Morilak DA, Barrera G, Echevarria DJ, Garcia AS, Hernandez A, Ma S, Petre CO (2005) Role of brain norepinephrine in the behavioral response to stress. Prog. Neuropsychopharmacol. Biol. Psychiatry 29, 1214–1224

Murillo-Rodríguez E, Di Marzo V, Machado S, Rocha NB, Veras AB, Neto GAM, Budde H, Arias-Carrión O, Arankowsky-Sandoval G (2017) Role of N-Arachidonoyl-Serotonin (AA-5-HT) in Sleep-Wake Cycle Architecture, Sleep Homeostasis, and Neurotransmitters Regulation. Front. Mol. Neurosci. 10, 152.

Murillo-Rodríguez E, Millán-Aldaco D, Di Marzo V, Drucker-Colín R (2008) The anandamide membrane transporter inhibitor, VDM-11, modulates sleep and c-Fos expression in the rat brain. Neuroscience 157, 1–11.

Myers B, McKlveen JM, Herman JP (2014) Glucocorticoid actions on synapses, circuits, and behavior: implications for the energetics of stress. Front. Neuroendocrinol. 35, 180–196.

Okuda S, Roozendaal B, McGaugh JL (2004) Glucocorticoid effects on object recognition memory require training-associated emotional arousal. Proc. Natl. Acad. Sci. U. S. A. 101, 853–858.

Oropeza VC, Mackie K, Van Bockstaele EJ (2007) Cannabinoid receptors are localized to noradrenergic axon terminals in the rat frontal cortex. Brain Res. 1127, 36-44.

Patel S, Roelke CT, Rademacher DJ, Cullinan WE, Hillard CJ (2004) Endocannabinoid Signaling Negatively Modulates Stress-Induced Activation of the Hypothalamic-Pituitary-Adrenal Axis. Endocrinology 145, 5431–5438.

Pava MJ, den Hartog CR, Blanco-Centurion C, Shiromani PJ, Woodward JJ (2014) Endocannabinoid Modulation of Cortical Up-States and NREM Sleep. PLoS One 9, e88672.

Pava MJ, Makriyannis A, Lovinger DM (2016) Endocannabinoid Signaling Regulates Sleep

Stability. PLoS One 11, e0152473.

Pudovkina OL, Kawahara Y, de Vries J, Westerink BH (2001) The release of noradrenaline in the locus coeruleus and prefrontal cortex studied with dual-probe microdialysis. Brain Res. 906, 38-45.

Ratano P, Everitt BJ, Milton AL (2014) The CB1 Receptor Antagonist AM251 Impairs Reconsolidation of Pavlovian Fear Memory in the Rat Basolateral Amygdala. Neuropsychopharmacology 39, 2529–2537.

Ratano P, Palmery M, Trezza V, Campolongo P (2017) Cannabinoid Modulation of Memory Consolidation in Rats: Beyond the Role of Cannabinoid Receptor Subtype 1. Front. Pharmacol. 8, 200.

Reyes BA, Szot P, Sikkema C, Cathel AM, Kirby LG, Van Bockstaele EJ (2012) Stress-induced sensitization of cortical adrenergic receptors following a history of cannabinoid exposure. Exp Neurol. 236, 327-335.

Roozendaal B, Castello NA, Vedana G, Barsegyan A, McGaugh JL (2008) Noradrenergic activation of the basolateral amygdala modulates consolidation of objectrecognition memory. Neurobiol Learn Mem. 90, 576-579.

Roozendaal B, McReynolds JR, McGaugh JL (2004) The Basolateral Amygdala Interacts with the Medial Prefrontal Cortex in Regulating Glucocorticoid Effects on Working Memory Impairment. J. Neurosci. 24, 1385–1392.

Roozendaal B, Okuda S, de Quervain DJ, McGaugh JL (2006a) Glucocorticoids interact with emotion-induced noradrenergic activation in influencing different memory functions. Neuroscience 138, 901-910.

Roozendaal B, Okuda S, Van der Zee EA, McGaugh JL (2006b) Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. Proc. Natl. Acad. Sci. U. S. A. 103, 6741–6746

Rueda-Orozco PE, Soria-Gomez E, Montes-Rodriguez CJ, Martínez-Vargas M, Galicia O, Navarro L, Prospero-García O (2008) A potential function of endocannabinoids in the selection of a navigation strategy by rats. Psychopharmacology (Berl). 198, 565–576.

Sano K, Koushi E, Irie K, Higuchi S, Tsuchihashi R, Kinjo J, Egashira N, Oishi R, Uchida N, Nagai H, Nishimura R, Tanaka H, Morimoto S, Mishima K, Iwasaki K, Fujiwara M (2009) Delta(9)-tetrahydrocannabinol enhances an increase of plasma corticosterone levels induced by forced swim-stress. Biol. Pharm. Bull. 32, 2065–7.

Santucci V, Storme JJ, Soubrié P, Le Fur G (1996) Arousal-enhancing properties of the CB1 cannabinoid receptor antagonist SR 141716A in rats as assessed by electroencephalographic spectral and sleep-waking cycle analysis. Life Sci. 58, PL103-10.

Sara SJ (2009) The locus coeruleus and noradrenergic modulation of cognition. Nat. Rev. Neurosci. 10, 211–223.

Schneider AM, Simson PE (2007) NAN-190 potentiates the impairment of retention produced

by swim stress. Pharmacol. Biochem. Behav. 87, 73–80.
Smarr BL, Jennings KJ, Driscoll JR, Kriegsfeld LJ (2014) A time to remember: the role of circadian clocks in learning and memory. Behav. Neurosci. 128, 283–303.
Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem. Biophys. Res. Commun. 215, 89–97.
Tapp WN, Holloway FA (1981) Phase shifting circadian rhythms produces retrograde amnesia. Science 211, 1056–8.
Tasker JG, Chen C, Fisher MO, Fu X, Rainville JR, Weiss GL (2015) Endocannabinoid Regulation of Neuroendocrine Systems. Int. Rev. Neurobiol. 125, 163–201.
Valenti M, Viganò D, Casico MG, Rubino T, Steardo L, Parolaro D, Di Marzo V (2004) Differential diurnal variations of anandamide and 2-arachidonoyl-glycerol levels in rat brain. Cell. Mol. Life Sci. 61, 945–950.
Winters BD, Tucci MC, DaCosta-Furtado M, (2009) Older and stronger object memories are selectively destabilized by reactivation in the presence of new information. Learn Mem. 16, 545-553.
Wolf OT, Atsak P, de Quervain DJ, Roozendaal B, Wingenfeld K (2016) Stress and Memory: A Selective Review on Recent Developments in the Understanding of Stress Hormone Effects on Memory and Their Clinical Relevance. J Neuroendocrinol.
Zeelenberg R, Wagenmakers E-J, Rotteveel M (2006) The Impact of Emotion on Perception. Psychol. Sci. 17, 287–29

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