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«ΝΕΥΡΟΕΠΙΣΤΗΜΕΣ»

Επίδραση των ανταγωνιστών των υποδοχέων της εκλυτικής ορμόνης της κορτικοτροπίνης στην αγχώδη συμπεριφορά και στις γνωστικές λειτουργίες του προμετωπιαίου φλοιού αρσενικών και θηλυκών μυών

Effects of Corticotropin Releasing Factor antagonists on anxiety-like behavior and prefrontal cortex-related cognitive functions in male and female mice

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1.1 ΠΕΡΙΛΗΨΗ

Οι ζωντανοί οργανισμοί αντιμετωπίζουν στρεσογόνους συνθήκες στις οποίες αποκρίνονται μέσω της ενεργοποίησης ποικίλων ενδογενών συστημάτων, όπως του συστήματος Υποθαλάμου-Υπόφυσης-Επινεφριδίων. Ο άξονας ΥΥΕ ρυθμίζεται κυρίως από τη δράση του εκλυτικού παράγοντα απελευθέρωσης της κορτικοτροπίνης (CRF). Οι νευρώνες που εκφράζουν CRF στον υποθάλαμο και άλλες εγκεφαλικές περιοχές ενεργοποιούνται έπειτα από στρεσογόνα ερεθίσματα και απελευθερώνουν τον CRF. Αυτός προσδένεται και ενεργοποιεί τους CRF1 υποδοχείς στον προμετωπιαίο φλοιό (PFC). Ευρήματα αναδεικνύουν την ιδιότητα του CRF να βελτιώνει τη μάθηση και τη μνήμη. Όμως, στη βιβλιογραφία τονίζονται οι διαφορετικές επιδράσεις του στρες στην αγχώδη συμπεριφορά και τη γνωστική λειτουργικότητα αρσενικών και θηλυκών υποκειμένων. Επομένως, ερευνήσαμε τις επιδράσεις του οξέος στρες περιορισμού και της ενεργοποίησης των CRF υποδοχέων στην αγχώδη συμπεριφορά και στις σχετικές με τον έσω προμετωπιαίο φλοιό (mPFC) σε ποντίκια των δύο φύλων τύπου C57BL/6. Αρσενικά και θηλυκά πειραματόζωα είτε υποβλήθηκαν σε δίωρο στρες περιορισμού (RS), είτε παρέμειναν στο κλουβί διαμονής τους πριν τη διεξαγωγή της δοκιμασίας Φωτεινού-Σκοτεινού Διαμερίσματος (LD) και τη δοκιμασία Διάκρισης Χρονικής Παρουσίασης Αντικειμένων (TOR). Επιπλέον, μία ακόμη ομάδα αρσενικών και θηλυκών μυών υποβλήθηκαν σε στερεοτακτική επέμβαση για την εμφύτευση κάννουλας-οδηγού στον Προμεταιχμιακό Προμετωπιαίο Φλοιό. Επίσης, σε μία υποομάδα των μυών όπου εμφυτεύθηκαν κάννουλες έγινε η χορήγηση ενός μη εκλεκτικού ανταγωνιστή των CRF υποδοχέων [α-helical CRF (9-41)] στην περιοχή-στόχο αμέσως μετά το στρες περιορισμού. Στη δοκιμασία Φωτεινού-Σκοτεινού Διαμερίσματος, οι θηλυκοί μύες της ομάδας στρες περιορισμού παρέμειναν για σημαντικά μικρότερη διάρκεια στο φωτεινό διαμέρισμα ($p=0,004$), σε σχέση με τους θηλυκούς μύες της ομάδας ελέγχου, ενώ τέτοιο αποτέλεσμα δεν προέκυψε για τις ομάδες των αρσενικών μυών. Ο Δείκτης Διάκρισης (DI) των αρσενικών μυών της ομάδας του στρες περιορισμού ήταν σημαντικά χαμηλότερος ($p=0,05$), σχετικά με αυτόν των μυών της ομάδας ελέγχου, στη Διάκρισης Χρονικής Παρουσίασης Αντικειμένων, ενώ ο ίδιος δείκτης ήταν όμοιος στις ομάδες των θηλυκών ζώων. Οι δείκτες Σκοτεινού/Φωτεινού Διαμερίσματος και Διάκρισης στους αρσενικούς μύες της ομάδας ελέγχου συγκριτικά με αυτούς των αρσενικών μυών όπου εμφυτεύθηκε κάννουλα δεν διέφεραν

σημαντικά. Σχετικά με τις δράσεις της χορήγησης α-helical CRF (9-41), οι αρσενικοί μύες της ομάδας ελέγχου και αυτής όπου εγχύθηκε το φάρμακο ενδοεγκεφαλικά, δεν διέφεραν ως προς το Δείκτη Διάκρισης. Το τελευταίο εύρημα υποδεικνύει την ιδιότητα της ουσίας να αναστέλει τις προκαλούμενες από στρες περιορισμού γνωστικές δυσλειτουργίες στα αρσενικά ζώα. Ο θηλυκός μυς που δέχθηκε τη χορήγηση α-helical παρέμεινε στο σκοτεινό διαμέρισμα για λιγότερη διάρκεια σχετικά με τα θηλυκά ζώα που υπέστησαν στρες περιορισμού και στα οποία δεν είχε εμφυτευθεί κάννουλα. Δεν παρατηρήθηκε κάποια επίδραση του οιστρικού κύκλου στα πειράματα αυτά. Συμπερασματικά, αναδείξαμε την επαγόμενη από στρες περιορισμού αγχώδη συμπεριφορά στην ομάδα των θηλυκών μυών που υπέστη στρες περιορισμού, και την γνωστική δυσλειτουργία των αρσενικών μυών που υπέστησαν στρες περιορισμού, όπως φάνηκε από τον χαμηλότερο Δείκτη Διάκρισης. Οι μελλοντικές μελέτες πρέπει να χαρακτηρίσουν τις επιδράσεις του α-helical CRF (9-41) στην αγχώδη συμπεριφορά και την επαγόμενη από το στρες γνωστική δυσλειτουργία, συλλέγοντας πειραματικά δεδομένα από μεγαλύτερο αριθμό ζώων.

1.2 ABSTRACT

Organisms face stressful conditions and respond to them through the activation of several endogenous systems, such as the Hypothalamus-Pituitary-Adrenal axis HPA axis is highly regulated by Corticotropin Releasing Factor (CRF). CRF-expressing neurons in hypothalamus and other brain regions are activated following stressful stimuli and release CRF. CRF binds to and activates CRF1 receptors in the PFC. Evidence shows the ability of CRF to improve learning and memory; however, findings emphasize the differential effects of stress in male and female anxiety and cognitive functioning. Thus, we investigated the effects of acute restraint stress and the effects of CRF receptor activation on the anxiety and medial-PFC cognitive functions in male and female C57BL/6 mice. Male and female animals were either subjected in 2-hour acute Restraint Stress (RS) conditions or remained in their home cage prior to the Light-Dark and Temporal Object Recognition (TOR) tasks. Further to the enquiry, another group of male and female mice were stereotactically implanted with a guide cannula in the Prelimbic PFC. In addition, a non-selective CRF receptor antagonist [(α-helical CRF (9-41)] was injected into the Prelimbic PFC of a subgroup of cannula-implanted animals immediately after the acute RS. In the Light-Dark test, RS female mice spent significantly less time in the dark compartment than the No-Restraint (NoRS) mice (p=0,004), while no such effect was found in male NoRS-RS groups comparison. The Discrimination Index (DI) of the male RS mice was significantly lower than that of the NoRS male mice in the TOR task (p=0,05), while the DI of NoRS and RS female mice was comparable. Dark/Light Index and DI of male control NoRS and cannula-implanted NoRS mice did not differ significantly. Regarding the α-helical CRF (9-41) effects, both NoRS and RS male α-helical injected mice showed similar DI, indicating the ability of α-helical to block the RS-induced memory performance. The female α-helical injected RS mouse spent less time in the dark compartment compared to the female control RS group. No effect of estrous cycle was found in these experiments. In conclusion, we showed the restraint stress-induced anxiety in the RS female group, and the impaired DI of the male RS mice. Future studies should unravel the effects of α-helical CRF (9-41) injection on anxiety and stress-induced cognitive dysfunctions gathering data from a greater number of experimental animals.

2. INTRODUCTION

2.1. Stress Response

All living organisms face stressors either physical or psychological in nature. The response to a stressful stimulus requires the activation of various endogenous systems such as autonomic, endocrine, immune, reproductive and cardiovascular system activation (Hillhouse & Grammatopoulos, 2006). Stressful stimuli lead to major changes in bodily responses originating from the activation of Autonomic Nervous System (ANS) and Hypothalamus-Pituitary-Adrenal Axis. The ANS responds fast, whereas the HPA axis causes more prolonged changes. Stress responses are critical for the survival of the organism but can contribute to the establishment of pathological states such as anxiety, depression and cognitive impairments, in case of chronically increased stress levels (Ketchesin et al., 2017).

In mammals, the activation of the several systems that respond in stressful circumstances is regulated by peptides such as Corticotropin Releasing Factor/Hormone (CRF or CRH) and urocortins (UCNs) (Hillhouse & Grammatopoulos, 2006). CRF, a 41-amino acid peptide was the firstly isolated from sheep hypothalami by Vale et al. (1981). In both mammals and non-mammals, CRF is expressed in brain regions such as hypothalamus, thalamus, hippocampus, nucleus accumbens, amygdala, bed nucleus of the stria terminalis, cortex, cerebellum and hindbrain (Owens & Nemeroff, 1991). Stressful stimuli activate the CRF expressing neurons in both hypothalamus and in other brain regions causing the release of CRF (Tsigos & Chrousos, 2002).

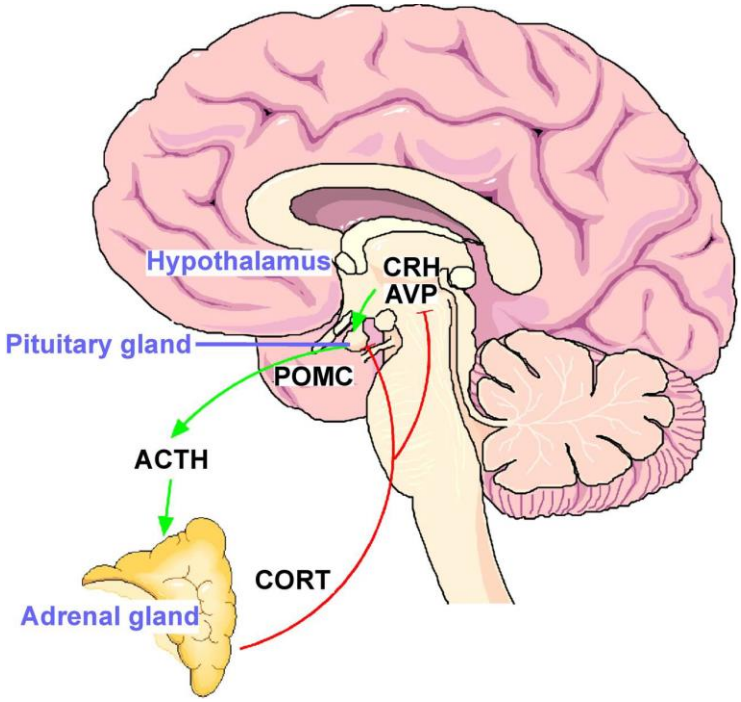

Figure 1: **The Hypothalamus-Pituitary-Adrenal (HPA) axis.** CRH is released from the hypothalamus into the anterior pituitary where it stimulates the release of ACTH into the blood stream. Adrenal gland -a target organ of ACTH, secrets cortisol in humans and corticosterone in rodents. The binding of glucocorticoids to their receptors in the hypothalamus and anterior pituitary acts as a mechanism of negative feedback for the activity of the HPA stress system (Murgatroyd & Spengler, 2011).

In mammals, paraventricular nucleus of the hypothalamus (PVN) -preoptic area (POA) CRH neurons in case of non-mammal vertebrates- signals for release of CRH that end up at the anterior pituitary through the hypophyseal portal system. The corticotropes of the anterior pituitary then release corticotropin (ACTH) into the systemic circulation which then acts on the adrenal cortex. There, cholesterol acts like the precursor molecule for the synthesis of steroid hormones. Thus, cortisol (or corticosterone in rodents) is secreted shortly after its synthesis. The increase of glucocorticoid levels is then balanced through the negative feedback it provides (Ketchesin et al., 2017).

The feedback systems of HPA axis is critical for maintaining hormone secretion at baseline level. Feedback systems act both acutely and chronically, either in response to a stressful stimulus or in a series of

days in order to keep hormone level at a baseline. The secretion of adrenal corticosteroids is attenuated through their binding to receptors present throughout the HPA axis (Keller-Wood, 2011). Thus, the role of the HPA axis is to act towards the minimization of deviation from homeostasis after the occurring of stressful stimuli (Handa et al., 1994).

Apart from the pituitary, upon stressful conditions CRF targets Locus Coeruleus (LC), the major noradrenergic nucleus of the brain (Valentino et al., 1983). CRF expressing neurons can modulate the activity of LC neurons both directly and indirectly; CRF axon terminals synapse with LC neuron dendrites as well as with afferent axon terminals of the LC (Van Bockstaele et al., 1996). CRF appears to act as a neurotransmitter in the LC, increasing the spontanous discharge rate of LC neurons (Valentino, 1988; Valentino et al., 1993). This increase leads to norepinephrine release increases in the PFC (Smagin et al., 1995).

LC receives innervation mainly from the nucleus paragigantocellularis (PGI and the nucleus prepositus hypoglossus (PH) (Aston-Jones et al., 1986). PGI neurons are stimulated by the activation of the sympathetic nervous system, while PH neurons take part in the control of eye movements. Both neuronal populations project to the LC, leading to increases in the PFC NE levels or to a shift of eye movements towards a new visual target (Berridge & Waterhouse, 2003).

LC acts as the main provider of noradrenergic neurotransmission in the neocortex and hippocampus. It consists of a “core” that contains two clusters of noradrenergic neurons each projecting to a different region (Mason et al., 1979). The rostral cluster projects to forebrain sites and the caudal cluster to segments of the spinal cord. Thus, LC is a critical brain region for maintenance of alertness and state-dependent cognitive processes (Berridge & Waterhouse, 2003).

2.2 CRF Receptors

CRF mediates its actions through binding at its two G-Protein Coupled Receptors: CRF1 and CRF2 which are expressed in the central nervous system (Stinnett et al., 2015). CRF1 receptor binds CRF with greater affinity than with the CRF2 type (Perrin et al., 1999). Apart from CRF receptors, glucoprotein CRH-Binding Protein (CRF-BP) binds CRH with affinity greater than its receptors (Seasholtz et al., 2002). CRF-

BP is highly expressed in the pituitary and its binding to CRF results in attenuation of ACTH release from the pituitary. It is therefore apparent that CRF-BP is able to reduce the CRF stimulatory activity upon its receptors in the pituitary (Westphal et al., 2006).

CRF1 receptors are expressed in several brain regions such as the cortex, olfactory bulb, pituitary, hippocampus, and cerebellum, as well as in the periphery in lower levels -notably in the adrenal cortex (Hauger et al., 2003). CRF1 receptor is the main CRF receptor subtype in the PFC (Chalmers et al,. 1995).

CRFR1 is the main mediator of signal transduction in the anterior pituitary (Perrin et al., 1993). The anterior pituitary can respond to hypothalamic CRF stimulation in several ways, dependent on the number of CRF1 receptors that expresses. The latter is influenced by the physiologic state of the organism (Panagiotakopoulos & Neigh, 2014). Notably, it has been reported that immobilization stress or CRF leads to reduction of the number of CRFR1 in the anterior pituitary (Hauger et al., 1988).

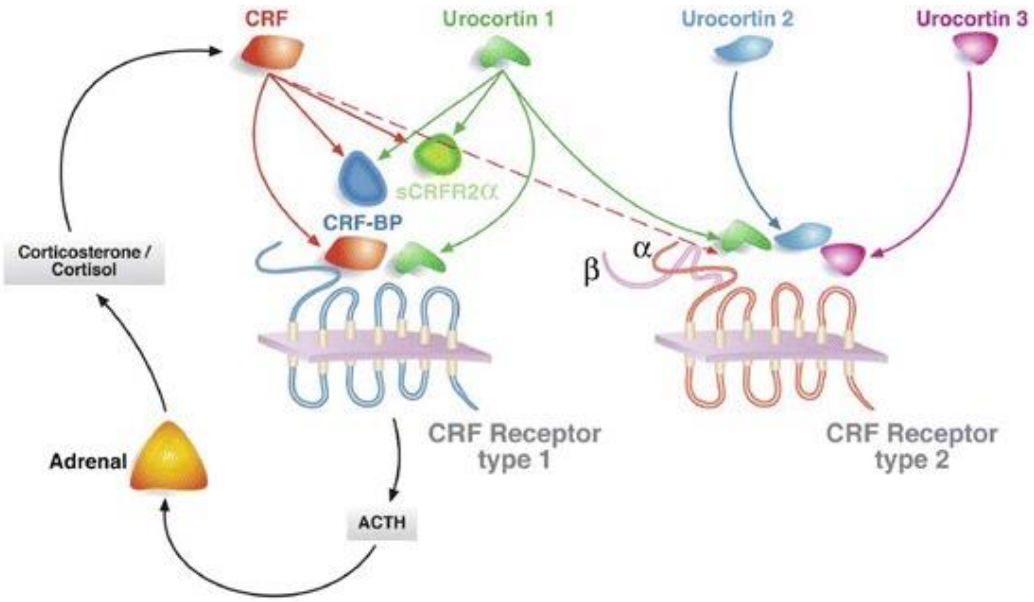

Figure 2: Schematic representation of mammalian CRF-urocortin family of peptides and their receptors. The dotted arrow-line is indicative of the lower affinity of CRF binding to the CRF2 receptor. The binding of CRF to the CRF1 receptors of the anterior pituitary causes the secretion of ACTH which targets adrenal glands among other organs. Cotisol or corticosterone is then secreted from the adrenal glands and provides negative feedback to the HPA system (Kuperman & Chen, 2008)

Upon binding to the CRFR1, CRF activates mainly Gs proteins that lead to the secretion of cyclic adenosine monophosphate (cAMP) through the activation of adenylcyclase (AC). cAMP then activates the cAMP-dependent protein kinase A (PKA), which in turn result in the activation of cell membrane L-type calcium channels (Kuryshev et al., 1996). The activation of CRFR1 can also lead to activation of Protein Kinase C (PKC) resulting in release of Ca2+ from intracellular stores through P-type Ca2+ channels into the cytoplasm (Dermitzaki et al., 2004). The increased intracellular Ca2+ that results from the activation of both PKA and PKC acts like a stimulator of ACTH sythesis and secretion from the pituitary (Panagiotakopoulos & Neigh, 2014). CRFR can also activate Gi, Go, Gq and Gz proteins, resulting in the activation of several protein kinases, such as PKC, PKB and MAP kinases (Grammatopoulos et al., 2001).

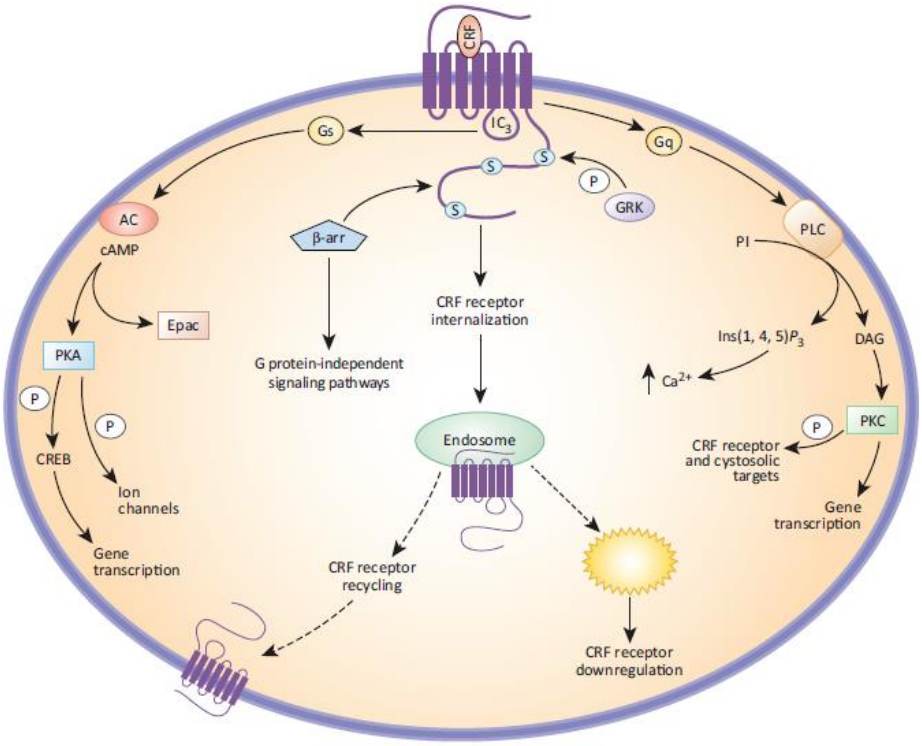

Figure 3: Schematic representation of the main signaling pathways that are associated with CRF1 receptor. The binding of CRF to the CRF1 receptor stimulates Gsα or Gq proteins, each triggering a distinct intracellular signaling cascade. The phosphorylation of the carboxy tail of the receptor by G-protein Receptor Kinases (GRKs) recruits β-arrestin 2 which initiates the internalization of CRFR1 into the endosomes (Valentino et al., 2013)

CRF2 receptor type is highly expressed in the periphery, although there is CRFR2 expression in a number of brain regions. In the brain, CRFR2 can be found in the lateral septum, bed nucleus of stria terminalis (BNST), ventromedial thalamus as well as the raphe nuclei, while in periphery CRFR2 are expressed in the heart, skeletal muscles, vasculature and in the gastrointestinal tract (Van Pett et al., 2000; Hauger et al., 2003).

2.3. Medial PFC related cognitive functions

Prefrontal cortex (PFC), a brain region highly correlated with control of emotion and cognition (Miller, 1999). PFC is essential for the implementation of several cognitive functions, such as working memory, attentional set shifting, inhibition control decision making and cognitive flexibility (Goldman-Rakic, 1996).

PFC consists of a dorsal and a medial part. The dorsal part of the PFC appears to be critical for working memory (Bangasser & Kawasumi, 2015). The function of the medial PFC is responsible for executive functioning, mediating among else, the integration of past experience and current inputs (Uribe-Marino et al., 2016). Furthermore, mPFC goal-directs behavior monitoring the changes in the environment (Azuar et al., 2014). Medial PFC also appears to be important for cognitive tasks such as recognition memory and discrimination of relative familiarity of individual stimuli (Barker et al., 2007).

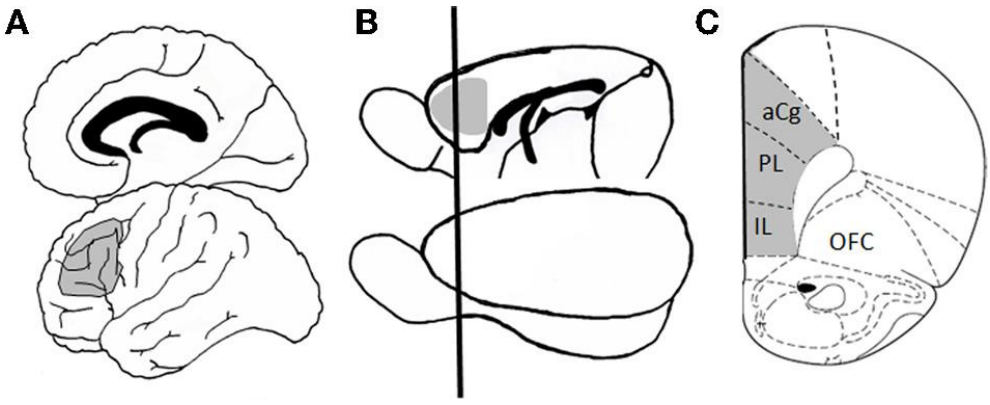

Figure 4: Prefrontal Cortex in the human and mouse brain. A. Schematic representation of dorsal lateral PFC (gray shaded area); B. Medial PCF, the dorsolateral PFC neuroanatomical homologue, is shaded in gray; C. Subdivisions of mPFC are shaded in gray: Anterior Cingulate Cortex (aCg), Prelimbic (PL) and Infralimbic (IL) cortex (Bizon et al., 2012)

Patients with stress-related psychiatric disorders appear to be impaired in terms of executive functioning. Therefore, their capacity for planning, cognitive flexibility, attention and working memory is attenuated (Bangasser & Kawasumi, 2015). Several cognitive processes are affected by stress: cognitive functioning -such as attention, working memory, inhibition control, that is mediated through the PFC seems to be affected by stressful stimuli (Arnsten & Goldman-Rakic, 1998).

Since mPFC is essential executive functions like working memory, attention, inhibition and decision making, its processes are disrupted by impairments of this region. Evidence shows that mPFC functiong can be disrupted by both acute and chronic stress (Maeng & Shors, 2013). Cognitive dysfunctions caused by stress have been shown to be related with the activation of CRH receptors by CRH (Wang et al., 2013).

2.4. CRF Effects on Behavior & Cognition

Several findings in the literature support the hypothesis that CRF action can affect behavioral and cognitive outcomes in humans as well as in rodents. In both rodent and human brain, CRF is able to affect learning and memory (Radulovic et al., 1999). At low doses, CRF does not induce anxiety-like behavior, but instead can modulate learning and memory (Behan et al., 1995). While high doses of CRF induce anxiety-

like behavior, its displacement of its binding protein is able to improve learning and memory without affecting anxiety levels (Radulovic et al., 1999).

Contarino et al. (1999) have shown that deletion of CRFR1 receptor leads to impaired cognition as well as anxiety-like behavior: the performance of CRFR1 deficient mice in both the Elevated Plus Maze task and the Black-White test box was attenuated, suggesting impaired spatial recognition memory and anxiety-like behavior. Increased anxiety-like behavior has also been reported in CRFR2 deficient mice (Bale et al., 2000). There has also been reported that the performance of early age-stressed rats in the novel-object recognition and in the Morris Water Maze tasks is improved following the administration of CRFR1 antagonist (Ivy et al., 2010). Cole et al. (2016) have shown disruption of sustained attention by CRF administration in both male and female rats in a dose-dependent manner. However, there were differences among rats in several phases of the estrous cycle: female rats in diestrous had similar sustained attention performance with male rats, whereas rats in proestrous or estrous had better performance.

There is evidence that the activation of CRFR1 receptors through CRF injection into the dorsal hippocampus before training of fear conditioning enhances learning. This effect was blocked when an unselective CRF antagonist was injected in this region, but not when a CRFR2-specific antagonist was injected. However, CRF injection in the intermediate septum attenuated learning, an effect that was blocked by a CRFR2-specific antagonist. In contrast, injection of CRFR2-specific antagonist solely in the lateral intermediate septum led to an enhancement of learning. The same effects were shown when the injection of CRF or its antagonists followed the training for fear conditioning, suggesting that CRF activation can influence memory acquisition as well as memory consolidation (Radulovic et al., 1999).

It has been reported that injection of an antagonist of CRFR2 at the lateral septum results in blockade of contextual fear conditioning impairment that is caused by acute restraint stress. Furthermore, the inactivation of CRFR2 has been shown to increase locomotor activity of acutely restraint stressed mice in the Open Field. When the fear conditioning training started 1 hour after the acute restraint stress condition and that delay was accompanied by an intra-hippocampal injection of CRFR1 antagonist, blockade of anxiety-like behavior and impairment of contextual fear memory was induced. When a CRFR2-selective agonist was injected intraventricularly (ICV), both anxiety-like behavior and impairment of fear memory were blocked, while the function of HPA axis remained intact. Thus, Ryabinin et al. (2008) suggest that anxiety-like behavior leads to the memory impairment. Impairments of temporal memory can be detected 6-8 hours after

the acute stress condition. The impairment of executive functions was blocked by the CRF1 deletion in the region, without however affecting the function of HPA axis (Uribe-Marino et al., 2016).

CRF can affect the arousal state of several brain regions through its actions in the LC neurons (Valentino et al., 2013). It has been reported that CRF microinjection in the LC leads to desynchronization of cortical electroencephalographic activity, indicating elevation in arousal (Curtis et al., 1997). Stress also leads to elevated arousal, an effect that can be reversed by CRF antagonist microinjection into the LC.

Restraint stress facilitates an increase the CRF gene expression in the PVN (Yk et al., 2011). Acute restraint stress can also lead to increased CRF mRNA expression in the PFC, while administration of CRF in PFC primary cell culture was shown to increase CRFR1 expression through MEK/ERK 1/2 pathway. The observed increase in CRF receptor expression might be due to a positive regulatory loop that exits between CRF and the CRF expressing cells (Yk et al., 2011). Evidence for increased CRHR1 mRNA expression in regions of PFC after acute restraint stress suggests the relation of this increase to the impaired executive functioning of the latter.

Acute restraint stress can differentially affect the various parts of memory such as acquisition, consolidation and retrieval memory components. In a study of Li et al. (2012), 1-hour restraint stress that started 75 minutes before the test session of both Object Recognition Task and Object Location Task led to disruption of the recognition memory retrieval. Furthermore, restraint stress of 1-hour immediately after the acquisition of memory can disrupt the consolidation of short-term into long-term memories. The disruption of both retrieval and consolidation recognition memory components was negatively correlated with the plasma corticosterone levels of mice.

Berridge and Dunn (1989) showed that CRF and restraint stress induced decrease in exploratory behavior is evident in hypophysectomised as well as in intact mice. Thus, the activation of the HPA axis by restraint stress or CRF is not essential for the reduction of locomotor activity in rats. Dunn and Swiergiel (1999) report that restraint stress decreased the number of entries in the Elevated Plus Maze (EPM) test in a similar way in wild-type mice, as well as CRF knock-out mice. These results suggest that another factor except CRF can mediate the effects of the latter.

Delivery of CRH directly in the medial PFC causes disruption of recency memory similar to that caused by acute restraint stress (Uribe-Marino et al., 2016). It is suggested that acute stress causes impairment in executive functions mediated by medial PFC through the CRHR1 activation and

phosphorylation of PKA and CREB (Uribe-Marino et al., 2016). Also, the increased PKC activation in the medial PFC of stressed rodents that appear to be impaired in terms of working memory functioning. There is also been shown that increased PKA activity is accompanied by disrupted executive functions (Kobori et al., 2015).

Acute stress can preferentially affect spatial memory of male or female animals; Conrad et al. (2004) showed facilitation of spatial memory in female rats, whereas spatial memory of males was found to be impaired, after being subjected to acute stress. The effect of acute stress on female rats was not affected by their estrus cycle phase, that was either proestrus or estrus during the experiment.

Intracerebrovesticular injection of CRF in mice elicited anxiety-like exploratory behavior decreasing the locommotor activity of the animals (Berridge & Dunn, 1986). ICV treatments that inhibit NE release or block α1-receptors caused an increase in exploratory behavior even in case of restraint stress conditions. This suggests that restraint stress must involve the activation of α1 adrenergic receptors (Berridge & Dunn, 1989).

2.5. α-Helical CRF (9-41)

α-Helical CRF (9-41) peptide is a non-selective CRF antagonist that has been used to elucidate the actions of CRF and its receptors. Physiological stress symptoms, such as hypertension, tacychardia, hyperthermia, become attenuated after ICV injection of α-helical CRF (9-41) in rats (Morimoto et al., 1993), while in unstressed rats α-helical CRF seems to induce no effects.

CRF ICV injection leads to reduction of time rodents spent in the open arms of the Elevated Plus Maze, while antagonist administration attenuates the CRF-induced anxiety-like behavior (Spadaro et al., 1990). CRFR antagonist α-helical CRF (9-41) has been shown to reverse the anxiety-like behavior that ICV CRF injections can elicit in additional studies. Berridge and Dunn (1987) showed that both CRF ICV injections and restraint stress reduced the time animals spent interacting with novel stimuli and that these effects can be reversed by a-hel CRF ICV injections in a dose-dependent manner.

Doses of CRF (0.1, 0.2 and 0.4μg) when administered ICV have been shown to induce anxiety-like behavior in rats, an effect that was blocked by α-helical CRF (9-41) ICV injection (Kumar & Karanth, 1996). Martins et al. (2000) showed that 4-hour restraint stress 24 hours before the Elevated Plus Maze test

decreased exploration in stressed rats, and that this effect was reversed by α-helical CRF (9-41) microinjection (0.5μg) into the periaqueductal gray.

A-helical CRF(9-41) has also reversed the anxiety-like behavior of ethanol withdrawn rats when administered (250ng) bilaterally into the central nucleus of the amygdala (Rassnick et al., 1993). Additionaly, α-helical CRF (9-41) could block the conditioned anxiogenic response caused by nicotine injection rats as shown by Tucci et al. (2003). However, ICV injections of α-helical may induce biphasic effects with respect to the dose delivered; low doses (5 and 25μg ICV) may cause no effect, while high doses (50μg ICV) of α-helical CRF (9-41) can lead to anxiety-like behavior with results similar to that of the CRF injection condition (Baldwin et al., 1991).

In a study using four strains of mice, α-Helical CRF (9-41) ICV injection of 25 or 50μg increased the time that BALB/C mice spent in the open arms of the Elevated Plus Maze, whereas had no effect in the performance of CF-1 and CD mice in the same test (Conti et al., 1994). When diazepam (1-4 mg/kg ip) was administered to the animals, time spent in the open arms of the EPM increased in all four strains. In the case of CRF ICV injection, the locomotor activity in a novel environment was attenuated in the four strain of mice used in the study (Conti et al., 1994).

2.6. Sex differences in stress response

There is a considerable amount of evidence showing sex differences in stress responses. In general, female rats seem to be more susceptible to the effects of HPA axis activation (Handa et., 1994). Many studies have shown sex differences with respect to stress responses, reporting increased corticosterone secretion in females following stress (Panagiotakopoulos & Neigh, 2014). There has also been evidence of prolonged HPA axis activation in female rats after acute stress conditions (Farabollini et al., 1991).

CRF1 receptor binding is higher in several cortical regions and in amygdala in female compared to male rats (Weathington & Cooke, 2012; Weathington et al., 2014). During even baseline conditions, corticosterone levels in female rats are higher than male rats. Corticosterone and ACTH levels of female rats show greater changes during each day (Handa et al., 1994). Furthermore, exposure to stressful stimuli leads

to higher glucocorticoid levels in female rats, an effect that appears to be related with the estrous cycle phase (Seale et al., 2004).

There has been evidence for sexual dimorphism regarding the expression levels of CRH-BP. Notably, expression of CRH-BP was found significantly increased in female murine pituitary in comparison to male murines (Speert et al., 2002). There also have been evidence for variation in CRH-BP expression in accordance to estrus cycle phase: 80% of CRH-BP was expressed in prolactin-expressing cells during proestrus in female rats (McClennen et al.,1998). Restraint stress has been shown to increase CRH-BP mRNA expression 3.2-fold in the pituitary of male mice but 11.8-fold in females. The increase in CRH-BP protein levels was also observed in pituitaries of females, notably 4-6 hours after the 30-minute restraint stress (Stinnett et al., 2015).

Anatomical differences have been reported between male and female rodents with respect to locus coeruleus (LC) cytoarchitecture, since the dendritic length of the LC neurons appears to be bigger in female than male rats (Bangasser et al., 2011). Under stressful conditions, CRF that is released in the Locus Coeruleus causes an increase in the firing rate of the LC neurons (Van Bockstaele et al., 1999). Because the LC is the region where norepinephrine cell bodies are found, the increase of their firing rate leads to enhanced arousal in several brain regions (Curtis & Valentino, 1994; Van Bockstaele et al., 1998). However, the same doses of CRF can lead to different results with respect to sex. Thus, low doses of CRF can result in enhanced LC neuronal activity in females, whereas in male animals similar doses do not induce any effects (Curtis et al., 2002; Bangasser et al., 2010).

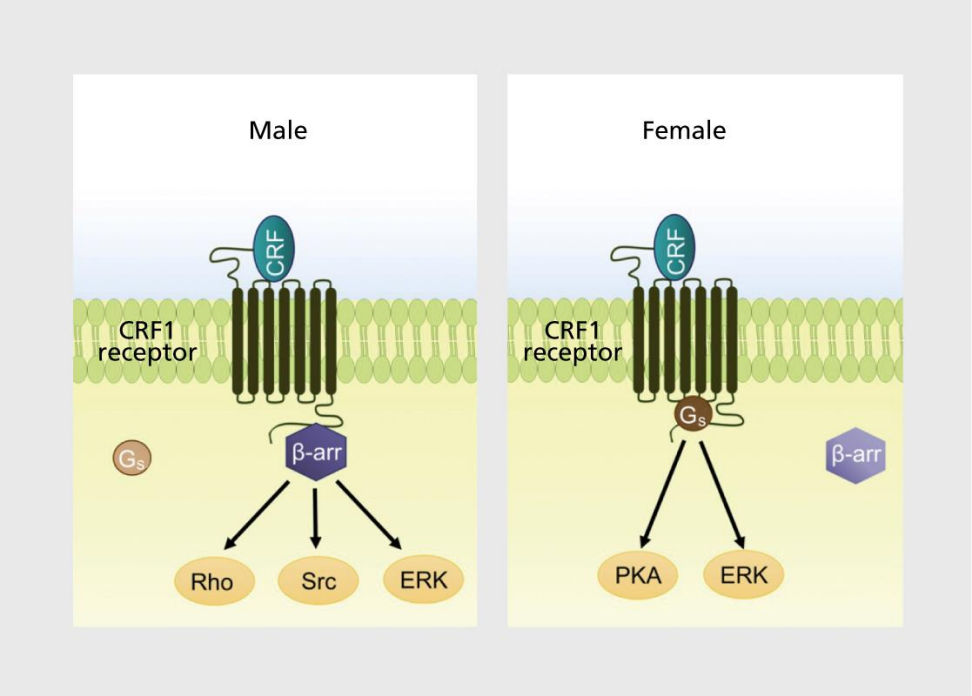

Figure 5: Schematic representation of sex-biased CRF1 receptor signaling. While CRF1 activation recruits β-arrestin2 in males, Gs stimulation is triggered in females., a difference that promotes distinct stress responses (Valentino & Bangasser, 2016)

CRFR1, the receptor type expressed in CL, appears to trigger different intracellular cascades in males and females upon activation. In female animals, the CRFR1 mainly stimulates G_s protein which activates the cyclic adenosine monophosphate (cAMP) – protein kinase A (PKA) pathway and result to increased LC neuronal firing (Hauger et al., 2009). In males, there is evidence that the CRFR1 activation stimulates βarrestin2 mediated signaling pathways, inducing the receptor internalization (Valentino et al., 2013a; Valentino et al., 2013b). While CRFR1 induces the cAMP-PKA signaling cascade in female rodents, in males CRF mainly activates β-arrestin2. Thus, the LC neuronal firing increase in response to low levels of CRF in females and not males could be explained by the differential activation of intracellular signaling pathways, which make female rodents more sensitive to CRF presence.

In baseline conditions, half of the CRF1 receptors can be found on the plasma membrane of LC neurons and the other half in the cytoplasm of male rats. When a stressor is applied, nearly 70% of the CRFR1 is found in the cytoplasm. However, there is evidence of the opposite phenomenon in female rats.

While in baseline conditions, most CRF1 receptors are cytoplasmic in LC neurons, stressful conditions lead the CRF1 receptors towards the plasma membrane. A possible explanation is the inability of β-arrestin2 to bind with the receptor (Valentino et al., 2013). Thus, due to the sex differences of CRF signaling, female rodents seem to be more sensitive to the actions of CRF and therefore less adaptable to excessive CRF availability (Valentino et al., 2013).

Sex differences are also lying in glucocorticoid secretion, with evidence supporting the elevated basal levels of corticosterone in female subjects (Viau et al., 2002). This hypothesis could explain findings such as the more robust despair-like behavior apparent in female rats on the Forced Swim Test following chronic mild stress but not after corticosterone treatment (Gobinath et al., 2016). However, the estrogen levels in females attenuate the number of corticosteroid receptors in the anterior pituitary, an effect that reduced the effectiveness of the negative feedback the glucocorticoids provide in the level of pituitary (Turner, 1990).

3. Aim of the study

In this study we decided to investigate the effects of CRF in medial PFC related cognitive functions in male and female mice. Animals undergone acute restraint stress prior to the Temporal Object Recognition Task to assess the recency memory performance. In addition, a group of mice undergone stereotactic surgery for the implantation of a guide cannula into the Prelimbic PFC to ensure the locomotor activity and cognitive functioning remained intact. Last, a subgroup of cannula – implanted animals was administered α-helical CRF (9-41) directly into the Prelimbic PFC in order to assess the effects of the CRF receptor antagonist on the mPFC related cognitive functioning.

4. Materials and methods

4.1 Animals

Wild type adult female (n=28) and male (n=26) C57BL/6 mice were used for this study. Animals were housed in groups of two and provided with standard mouse chow and water ad libitum, under a 12-hour light/dark cycle with controlled temperature (23±1°C). Approximately 8 days before the behavioral testing, all mice were handled by the experimenter every day for 5 minutes. This would reduce stress associated with handling with the experimenter during testing.

4.2 Drugs

α-helical CRF(9-41) (2,5μl) and Fast Green staining (10μl) were added to normal aCSF solution (37,5μl) making a stock preparation. From this mixture, 1μl was microinjected into the Prelimbic Prefrontal Cortex of each mouse in a rate of 30 seconds.

4.3 Microinjection

The peptide was microinjected in the prelimbic PFC of mice using a syringe with a long easily bent needle (Fig: 6) placed on a mini-pump. The total volume of the drug (1μl) was administered over 30 seconds by inserting the syringe needle into the guide cannula. The injection needle remained in place for additional seconds before it was removed, to prevent diffusion of the drug.

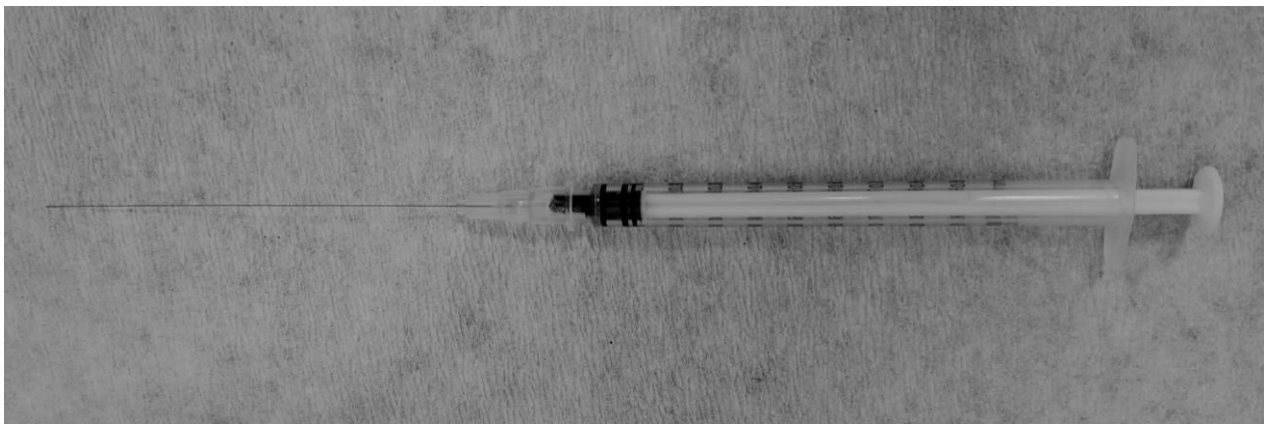

Figure 6: Syringe used for α-helical CRF (9-41) injection into the Prelimbic Cortex of mice.

4.4 Stereotactic surgery

Mice (n=9) undergone surgery weighted 23-28gr and were ???? months old. The subjects were deeply anaesthetized with a ketamine (1.5μl/gr i.p.), xylazine (1μl/gr i.p.) and sterile saline (1μl/gr i.p.) mixture before they were placed onto the stereotactic apparatus. In addition, lidocaine HCL (Xylocaine ®, 5%) was applied locally immediately after the opening of the scalp. The eyes were moistened with Vaseline® jelly throughout the procedure. The cannula was implanted at the Prelimbic Prefrontal Cortex of the right hemisphere according to Paxinos & Franklin (2012) coordinates (AP:+1.8, ML:-0.4, DV:-2.2). Two additional holes were drilled through the skull for placement of anchor screws that ensured better adherence of the skull cap. The cannula was lowered after the anchor screws placement. The exposed cranium was covered by acrylic polymer (Kallocryl® CPGM red), which secured the guide cannula in place and encasing the head of the anchor screws. After the cement dried, sutures were stitched and the mice were placed in heated cages for ~4-5 hours to allow recovery from the effects of anesthesia. Afterwards, the mice were administered with analgesic (Diclofenac, 5ml/g ip) and were returned to the animal house for one week recovery before the start of the handling by the experimenter.

Figure 7: Microinjection pump (CMA/100) that was used for the injection of α-helical CRF (9-41). The drug was released at a rate of 1μl/30sec.

4.5 Restraint stress

2 hours prior to the Light-Dark Task, half the mice were placed individually in restraint chambers, whereas the control mice stayed at their home cages for two hours. The restraint stress procedure took place in a different room than the animal house.

4.6 Behavioral testing

4.6a Light-Dark Task

After 2 hours of restraint stress/control conditions, each mouse was placed in the Light-Dark apparatus for anxiety like behavior assessment. The apparatus consists of two identical chambers -one dark and covered and one light and open, which are connected to each other through an open door (Figure 8). Firstly, the mice were placed in the dark chamber and were left to move freely between the two chambers. Latency and time spent in each chamber were measured for 5 minutes.

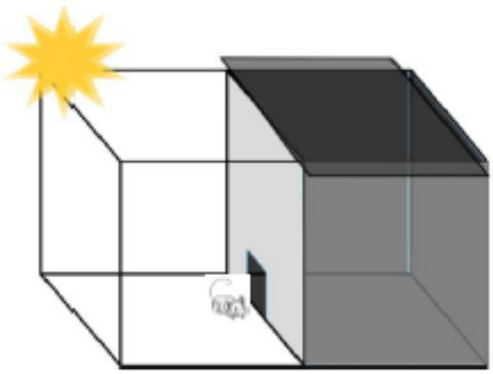

Figure 8: Light-Dark Apparatus.

4.6b Temporal Object Recognition (TOR) Task

The behavioral testing took place in an open field apparatus (40x40cm). For three consecutive days prior to the experiment, mice were individually placed in the open field apparatus for 15 minutes each day, in order for them to be habituated with the apparatus. Before the placement of each mouse in the arena, the Open Field apparatus was cleaned thoroughly using ethanol (70%). Habituation sessions were recorded using a web camera.

After 3 days of habituation, followed the experimental procedure. The TOR task consists of two 5-minute sample and one 5-minute test sessions with a 25-minute intertrial interval. In each sample session, 2 identical objects were used. The two identical objects for each sample session were placed on opposite sides of the apparatus, leaving 8cm from the walls. Each mouse was placed in the center of the arena and left to freely explore the objects for 5 minutes. 25 minutes after the 1st sample session, the animals were placed again in the OF apparatus for a 5-minute exploration of a different set of identical objects (second sample trial). For the test session that followed 25 minutes after the 2nd sample trial, one object from the 1st and one object from the 2nd trial were used. Again, mice were left to freely explore the objects for 5 minutes. All sample and test sessions were recorded using a web camera. Discrimination Index (Time spent exploring familiar object/Time spent exploring recent object) and total time exploring the two objects (Exploration Index) were measured for each test session.

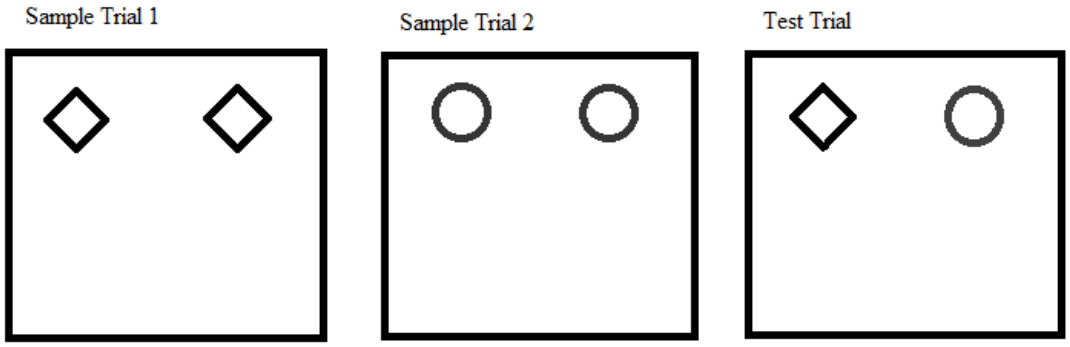

Figure 9: Temporal Object Recognition Task. Identical objects were used for Sample Trials, while in Test Trial an object from each of the Sample Trials was used.

4.8 Vaginal smear collection

Every habituation and experiment day, vaginal smear was collected from female mice. A latex bulb was placed on the end of a sterile pipette 200ul tip that was then filled with approximately ~100ul double distilled water (ddH_2O). The end of the ddH_2O tip was carefully placed at the opening of the vaginal canal as not to penetrate the orifice. Gentle depressions (4-5 times) of the bulb expelled a quarter to half of the volume of water (~25-50 μl) at the opening of the vaginal canal. The ddH_2O that withdrew back into the tip containing vaginal smear was placed onto a glass slide. Once dry, the smears were stained in order to identify the estrous cycle phase of the mice.

4.9 Cytological staining of vaginal smear

Firstly, the slides were placed in a coplin jar containing Crystal Violet staining for 1 minute. Then, they were washed twice with double distilled water (ddH_2O) for 1 minute. The excess ddH2O was removed from the slides using a tissue paper and the slides were cover-sliped with glycerol (15ul) and kept at room temperature for examination. After the cytological staining, images were obtained with a light microscope using the 10x and 25x objective lenses.

4.10 Histological verification of cannula placement

Immediately after the behavioral experiments, the animals were sacrificed by cervical dislocation and the brains were removed and put in PFA 4% solution. 24 hours later, the brains were removed from the PFA solution and were put in PBS solution after 3x 5-minute PBS washes. The brains were dissected by Vibratome and 40μm prefrontal cortex slices were Nissl stained.

4.11 Statistical Analysis

Preference of animals for Light/Dark compartments (ms in Dark and ms in Light compartment) and Latency (ms until first exodus into the Light compartment) were analyzed by the Student's t-test for each experimental group. Preference of animals for objects (ms mice spent exploring each object) and total exploration time were also analyzed by the Student's t-test for each experimental group.

5. RESULTS

5.1. Effects of Restraint Stress on Light-Dark and Temporal Object Recognition tasks

Restraint-stressed female mice spent significantly more time in the dark compartment of the Light-Dark apparatus compared to the unstressed female animals (p=0,004). This effect indicates higher anxiety-like behavior levels in the RS group (Fig:10a). Furthermore, both RS male (p=0,37) and female mice (p=0,18) spent similar amount of time in the dark compartment until their first exodus to the Light compartment, showing comparable latency scores (Fig:10b).

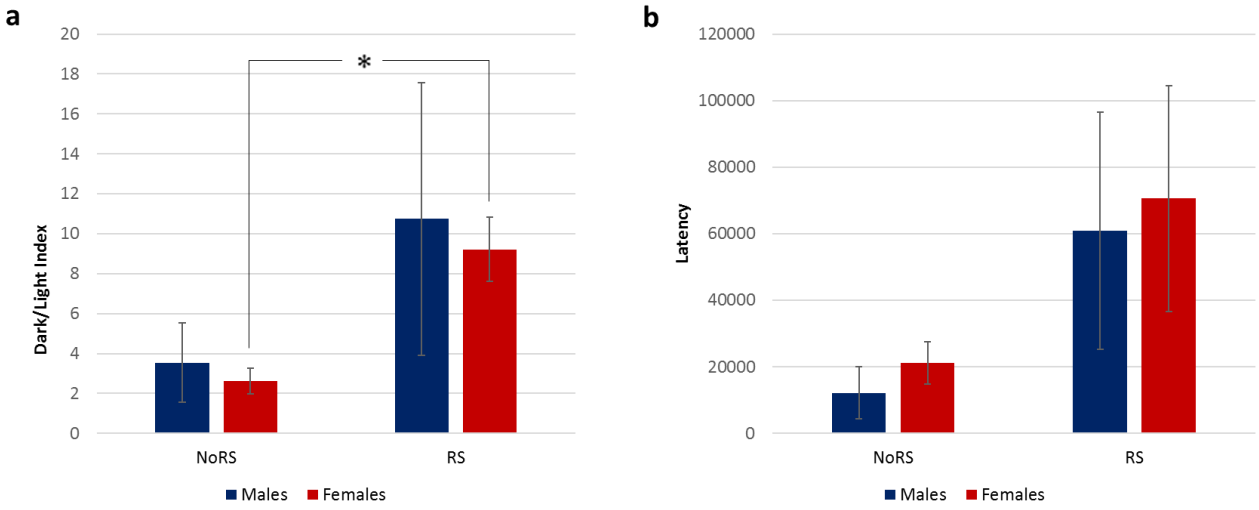

Figure 10: Effects of Restraint Stress on Light-Dark Task. (a) NoRS female mice spent significantly less time in the dark compartment of the Light-Dark apparatus (p=0,004). Male RS mice had no significant differences (p=0,89). (b) Latency scores of NoRS and RS male groups did not differred significantly (p=0,37), an effect that remained similar in female groups with respect to latency (p=0,18). (a) NoRS male group n=3, NoRS female group n=5, RS male group n=3, RS female group n=5. (b) NoRS male group n=2, NoRS female group n=5, RS male group n=3, RS female group n=5.

As shown in the Temporal Object Recognition task performance, the Discrimination Index of the male RS groups (Fig:11a) was significantly lower compared to the DI of unstressed male mice (p=0,05). The

exploration index of NoRS male and female groups (Fig:11b) was found to be similar to that of the RS male and female groups (p>0.05). These effects suggest that RS male animals show lower discrimination ability between older and more recent objects in the test trial.

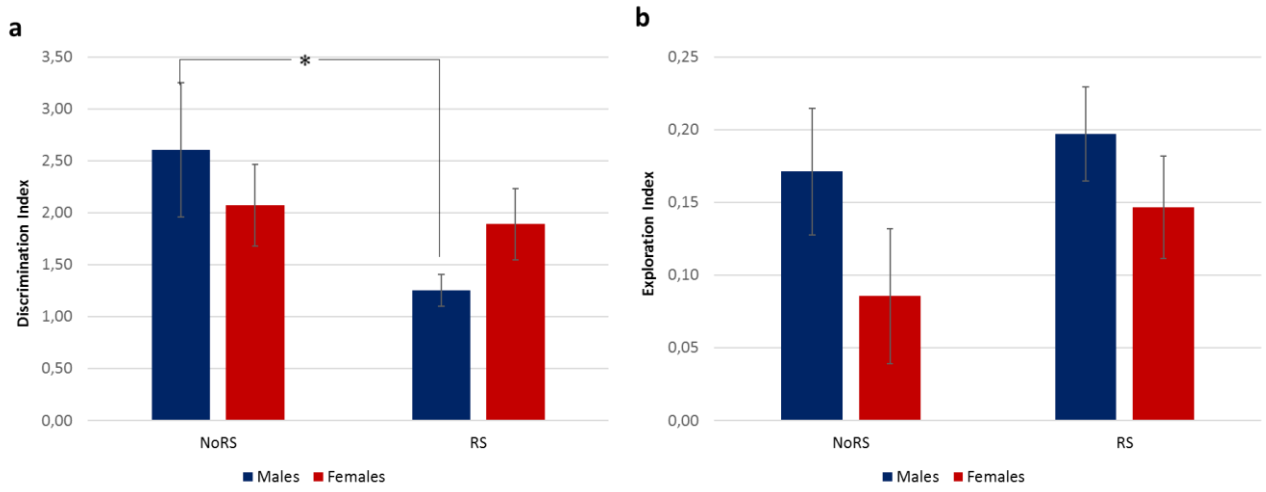

Figure 11: Effects of Restraint Stress on Temporal Object Recognition Task (a) Male NoRS and RS differed significantly with respect to the DI (p=0,050928141), while DI was similar between NoRS and RS female groups (p=0,73). (b) Exploration Index was comparable between NoRS and RS male mice (p=0,63), as well as in NoRS and RS female mice (p=0,39). (a) NoRS male group n=5, NoRS female group n=13, RS male group n=7, RS female group n=12.

5.2. Effects of stereotactic surgery and cannula implantation on Light-Dark and Temporal Object Recognition tasks

To reveal the potential implications of the implantation of cannulas on the Light-Dark and Temporal Object Recognition tasks performance, two groups of mice were compared. Male Control NoRS mice and male NoRS cannula-implanted mice were put through Light-Dark and Temporal Object Recognition tasks. No significant differences were found between the two groups in respect to Dark/Light Index (p=0,37) and Latency scores (p=0,49) (Fig: 12), suggesting similar levels of anxiety.

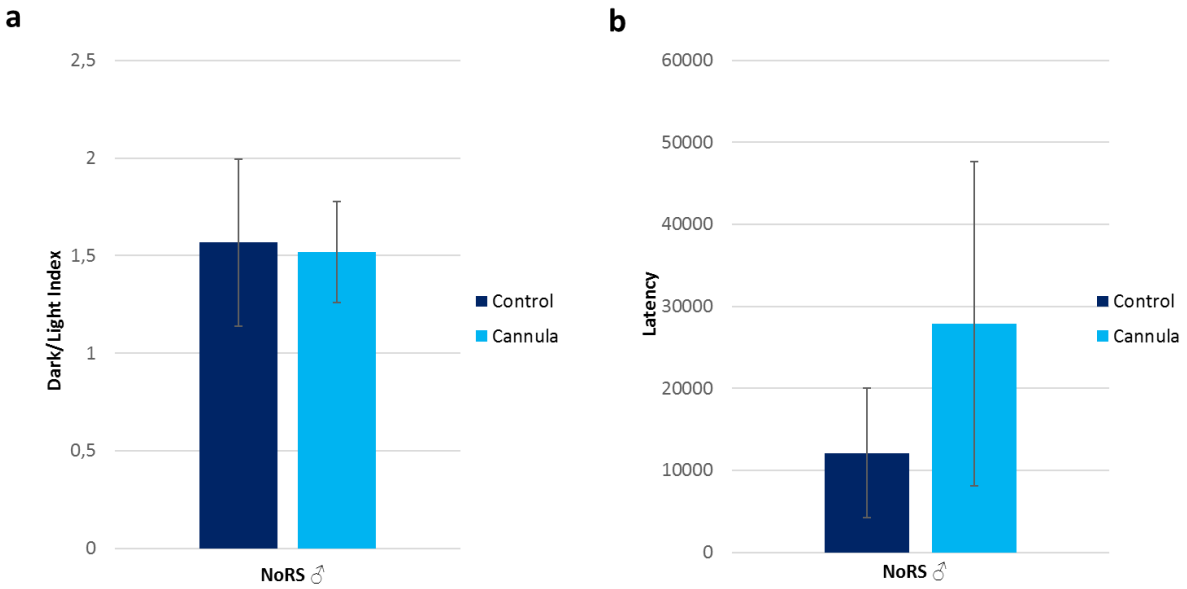

Figure 12: Effects of stereotactic surgery and cannula implantation on Light-Dark and task. (a) No significant differences were found between Control and Cannula-implanted NoRS male mice in the Dark/Light Index (p=0,37). (b) Latency scores were as well similar between the two groups (p=0,49). (a) Control NoRS male group n=3, Cannula-implanted NoRS male group n=3 (b) (a) Control NoRS male group n=2, Cannula-implanted NoRS male group n=3.

Temporal Object Recognition performance was also similar as the two groups showed comparable Discrimination (p=0,21) and Exploration (p= 0,41) Indexes. Results emerge from the TOR task suggest that

the stereotactic surgery procedure and cannula implantation do not significantly affect the discrimination ability or exploration of the animals (Fig: 13).

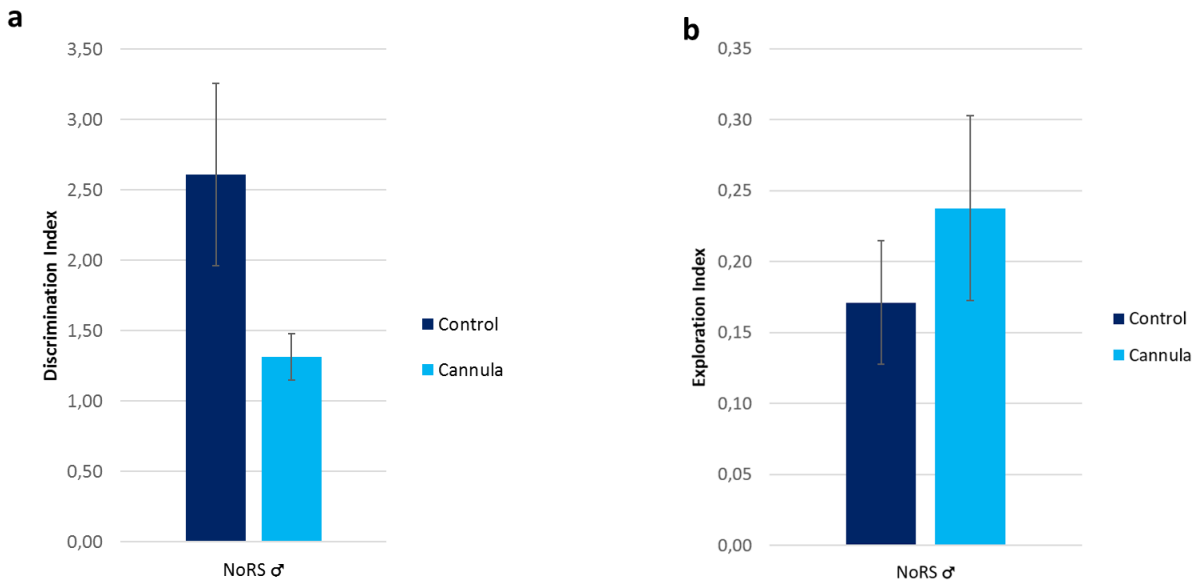

Figure 13: Effects of stereotactic surgery and cannula implantation on Temporal Object Recognition task. (a) DI was comparable between the Control and Cannula group (p=0,21). (b) While Exploration Index was also similar in the two groups (p= 0,41). (a) Control NoRS male group n=6, Cannula-implanted NoRS male group n=3.(b) Control NoRS male group n=6, Cannula-implanted NoRS male group n=3.

5.3. Effect of α-helical CRF (9-41) injection on Light-Dark and Temporal Object Recognition tasks

Injections of α-helical CRF (9-41) were performed to a subgroup of the Cannula-implanted animals following acute RS and prior to behavioral testing in order to study the potential anxiolytic and cognition-enhancing effects of CRF antagonism. The male unstressed α-helical-administered mouse showed a Dark/Light Index comparable to that of the unstressed male and female control groups, although its latency score was higher compared to the other unstressed control groups. The female RS α-helical-administered

mouse had lower Dark/Light Index and higher Latency score than the male and female RS groups, respectively.

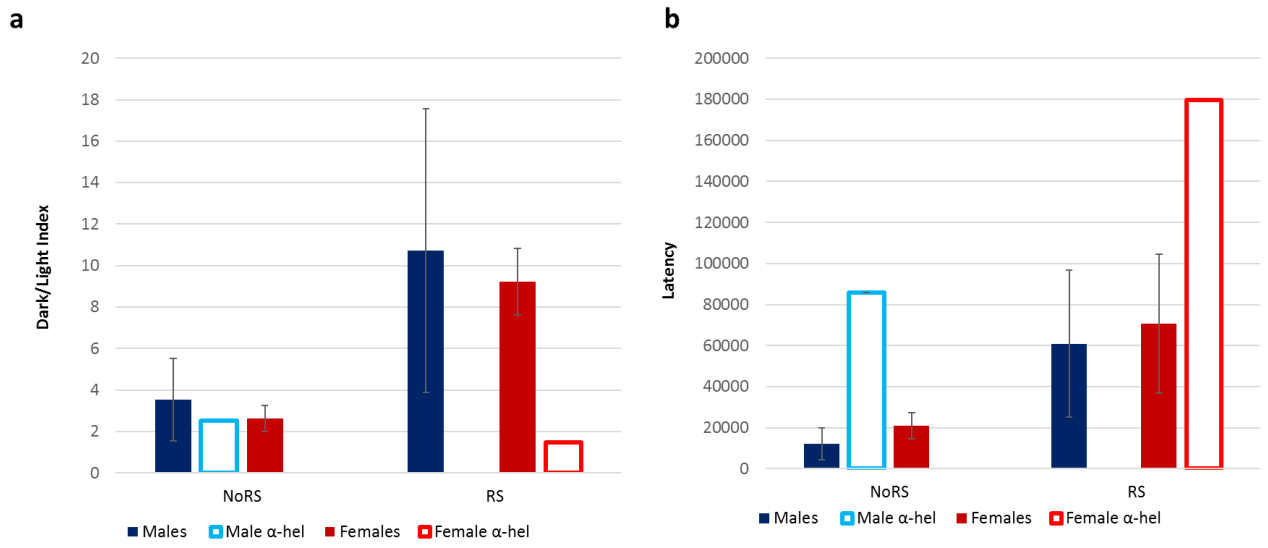

Figure 14: Effect of α-helical CRF (9-41) injection on Light-Dark task. (a) Comparison of Control NoRS male and female groups with the α-helical-administered male animal showed no difference. However, the α-helical-administered female mouse D/L Index was low compared to the Control RS female group. (b) Latency score of α-hel male mouse was higher than the latency score of both male and female control NoRS groups, whereas α-hel female mouse had a high latency score compared to the other RS male and female control groups. (a) Control NoRS male group n=3, α-hel injected NoRS male mouse n=1, Control NoRS female group n=5. Control RS male group n=3, Control RS female group n=5, α-hel injected RS female mouse n=1 (b) Control NoRS male group n=2, α-hel injected NoRS male mouse n=1, Control NoRS female group n=5, Control RS male group n=3, Control RS female group n=5, α-hel injected RS female mouse n=1

In the Temporal Object Recognition task, the DI of the male unstressed α-helical-administered mouse was similar to that of the Control NoRS male group, although its exploration index was lower. Also, the DI of the male RS α-helical-administered mouse was higher compared to the Control RS male group, and similar to the DI of the NoRS α-hel administered animal. However, the exploration index of the α-helical RS mouse was lower than that of the Control male RS group. Despite the acute stress condition, male α-helical mice showed similar DI scores. Furthermore, the DI and Exploration Indexes of the female RS α-hel injected mouse were comparable to these of the male RS α-hel administered mouse.

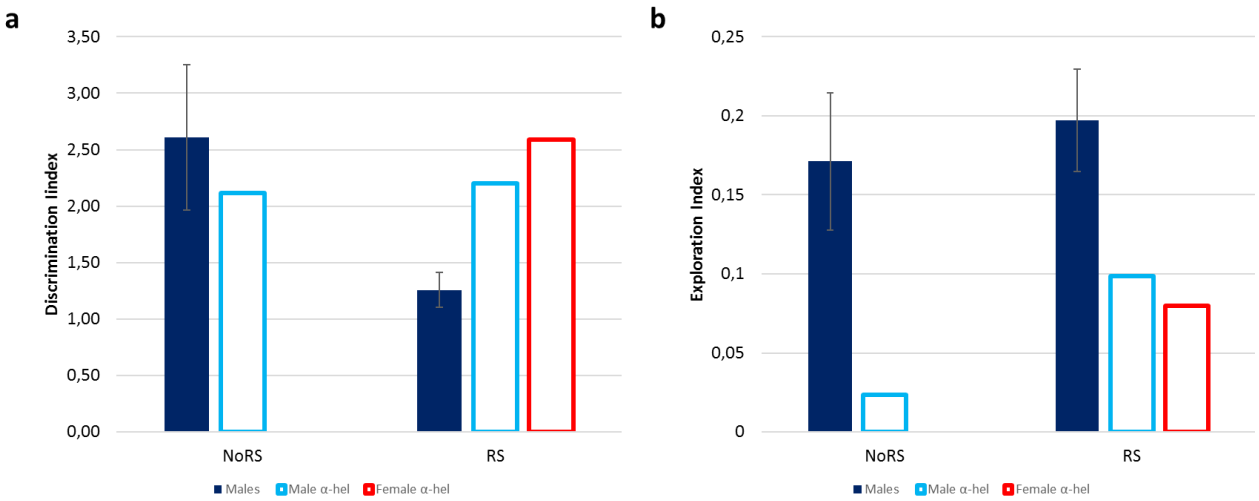

Figure 15: Effect of α-helical CRF (9-41) injection on Temporal Object Recognition task. (a) DI of α-hel NoRS male mouse was similar to that of the control NoRS male group. However, DI of α-hel RS male mouse was higher than the DI of the control RS male group and similar to that of the α-hel RS female mouse. DI of NoRS and RS α-hel male mice were similar. (a) Control NoRS male group n=6, α-hel injected NoRS male mouse n=1, Control RS male group n=7, α-hel injected RS male mouse n=1, α-hel injected RS female mouse n=1 (b) Control NoRS male group n=6, α-hel injected NoRS male mouse n=1, Control RS male group n=3, α-hel injected RS male mouse n=1, α-hel injected RS female mouse n=1

5.4. Histology

Figure 16: Representative images of histological verification of cannula implantation. PFC slices exhibiting traces of cannula-induced leisure in the mPFC.

5.5 Effect of estrous cycle phase on anxiety-like behavior and Temporal Object Recognition Discrimination Index

An effort was made to correlate the estrous cycle phase with the anxiety-like behavior and the cognitive functioning. Thus, the performance of a subgroup of female mice in the Light-Dark and TOR tasks were assessed in respect to each animal's estrous cycle phase. The performance of female unstressed and stressed female mice is shown in Fig.18 and 19. NoRS mice in proestrous and diestrous showed higher latency compared to the metestrous mouse, whose latency score was the lowest (Fig: 18a).

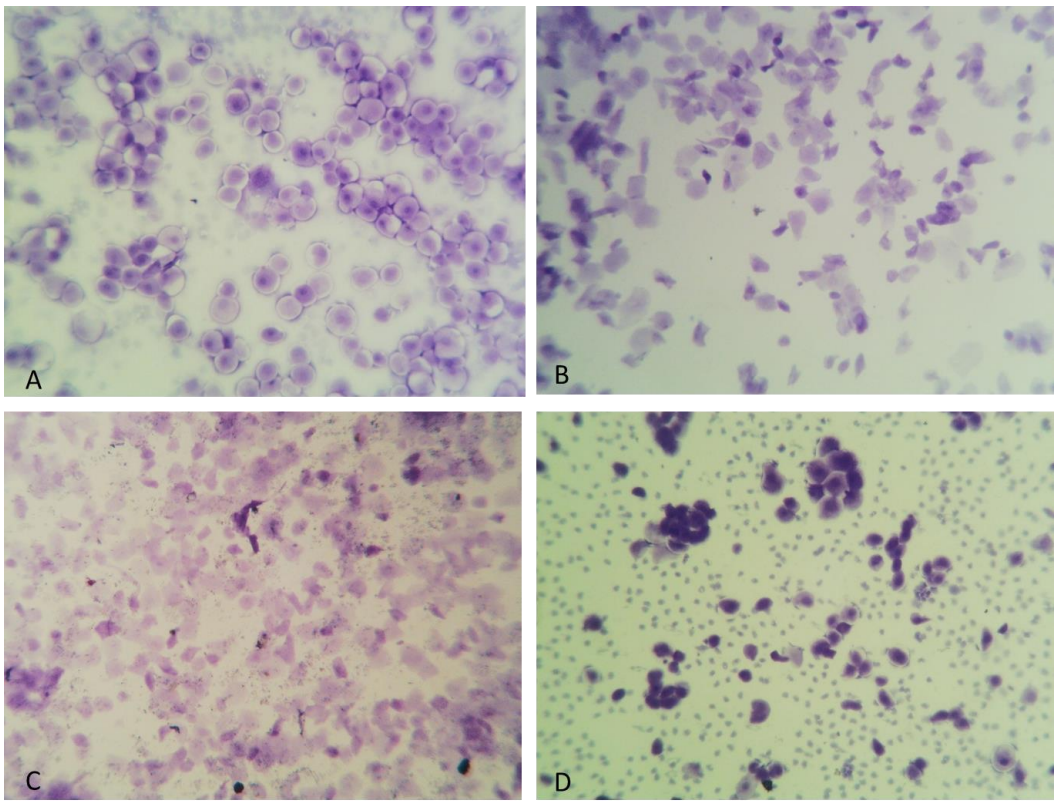

Figure 17: Vaginal cytology representing each phase of estrous. Stages of estrous cycle include Proestrous (A), Estrous (B), Metestrous (C) and Diestrous (D).

However, the Dark/Light Index of estrous animal was the highest of all other cycle phases (Fig:18b). In the TOR task, the Exploration Index of proestrous and metestrous mice was lower compared to the estrous and diestrous one, with the estrous mouse exhibiting the highest Exploration Index score (Fig:18c). In relation to the Discrimination Index, it was higher in Proestrous, Estrous and Diestrous mice, while lower in the Metestrous animal (Fig:18d). The highest DI corresponding to the Diestrous mouse.

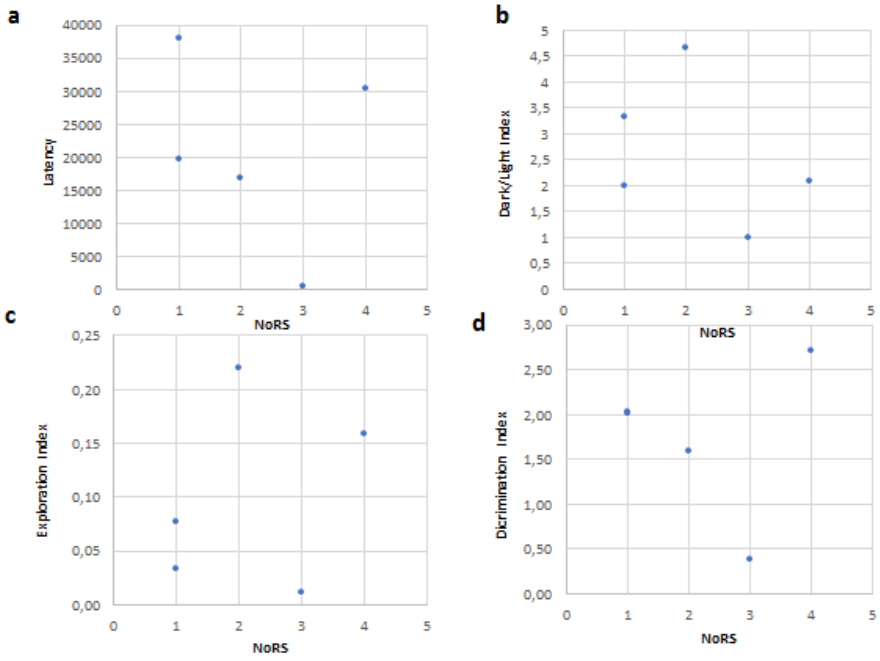

Figure 18: Effect of estrous cycle phase of unstressed female mice on anxiety-like behavior and Temporal Object Recognition task. Representation of Latency scores (a), Dark/Light (b), Exploration (c) and Discrimination Indexes of NoRS female mice. On the X-axis; 1: Proestrous, 2: Estrous, 3: Metestrous and 4: Diestrous.

In contrast, the stressed female mice in estrous exhibit higher latency scores than mice in proestrous and metestrous (Fig:19a). Mice in estrous and metestrous however had higher Dark/Light Index than that of proestrous mouse (Fig:19b). As shown by their performance in the TOR task, Exploration Index of mice in proestrous was the highest (Fig:19c). One of the proestrous animals had the highest DI of all cycle phase mice. Proestrous mice and one of the metestrous mice also showed high DI, whereas DI of the other estrous and metestrous mice was low (Fig:19d).

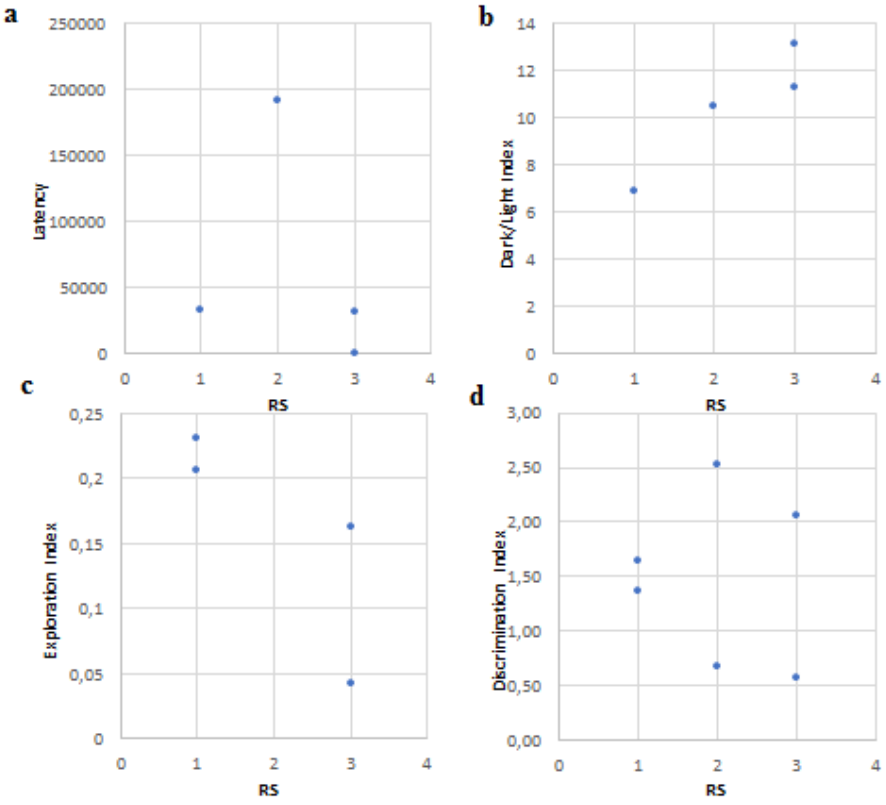

Figure 19: Effect of estrous cycle phase of Restraint female mice on anxiety-like behavior and Temporal Object Recognition task. Representation of Latency scores (a), Dark/Light (b), Exploration (c) and Discrimination Indexes of RS female mice. On the X-axis; 1: Proestrous, 2: Estrous, 3: Metestrous and 4: Diestrous.

6. Discussion

In this study, the effects of acute restraint stress on anxiety-like behavior and mPFC related cognitive functions were investigated in male and female mice. Half of the male and female animals undergone 2-hour Restraint Stress prior to the behavioral testing, while the control animals remained in their home cage. Then, their performance at the Light-Dark and Temporal Object Recognition tasks was assessed. Furthermore, the effects of stereotactic surgery and cannula implantation on anxiety and mPFC related cognitive functioning were assessed in male stressed and unstressed mice. Last, we investigated the effects of α-helical CRF (9-41) injection on anxiety and cognitive functions of a subgroup of Cannula-implanted animals.

Concerning the effect of acute restraint stress on the anxiety and mPFC-related cognitive functioning, we found significant differences in the Dark/Light Index between the unstressed and stressed female mice. This finding is supported by literature, as several researchers have underlined the increased sensitivity of female subjects in the effects of stress on anxiety-like behavior (Handa et., 1994; Curtis et al., 2002; Bangasser et al., 2010, Panagiotakopoulos & Neigh, 2014). Also, no significant differences were found between male unstressed and stressed groups in anxiety-like behavior, an effect that supports the anxiety resistant profile of male subjects.

However, a significant difference was found between male stressed and unstressed groups with respect to Discrimination Index in the TOR task. This suggests that acute stress attenuated recency memory of male mice. No differences were found in the female NoRS and RS groups, that showed similar levels of recognition memory. The level of stress had no significant effect in the exploration index in all male and female groups. Given that CRF promotes learning and memory (Radulovic et al., 1999), the sex biased intracellular signaling pathways of CRF1 receptors might underlie the attenuation of memory performance of stressed male mice. While in females CRF1 activation stimulates Gs proteins, in males activation of CRF1 receptor recruits β-arrestin2 which results in internalization of the receptor. Thus, CRF effectiveness in male animals might be decreased compared to female subjects.

To reveal the potential effects of stereotactic surgery and cannula implantation on anxiety, locomotor activity and cognitive functioning of animals, we compared the performance of cannula-implanted and control stressed and unstressed mice in the Light-Dark and TOR tasks. Light-Dark test performance did not show significant differences between control and cannula-implanted groups, suggesting that anxiety levels

are not affected by stereotactic surgery and cannula implantation. In the TOR task, no significant differences were found between the control and cannula-implanted groups in Discrimination and Exploration Index, although cannula-implanted mice DI was lower than that of the control group. This effect could be a result of the limited number of subjects (n=3) the cannula-implanted group consists of.

Next, the effects of peptide α-helical CRF (9-41) injection into the Prelimbic PFC were evaluated in a subgroup of the cannula-implanted animals. The Light-Dark task showed similar levels of anxiety among unstressed control male group and α-hel injected male and female mice, although the α-hel injected male animal had greater Latency score compared to the unstressed control male group. Stressed α-hel injected female mouse showed low Dark/Light Index compared to the stressed control female group, although it had greater latency score. This effect suggests that the stressed α-hel injected female animal had lower level of anxiety compared to the RS female group. Specifically, the Dark/Light Index of the α-hel injected RS female animal was comparable to that of the unstressed control female animals. Berridge and Dunn (1987) have also shown the reversal of the restraint stress effects by α-helical CRF (9-41) ICV injection in male CD-1 mice.

Notably, regarding the DI in the TOR task, the α-hel injected male animal showed a DI score similar to that of the unstressed male control mice and to that of the RS α-hel injected female mouse. What is more, DI of stressed α-hel injected male mouse was similar to that of the unstressed α-hel injected male animal, even if the DI of RS control male group is lower than that of its respective NoRS group. Regarding the Exploration Index of the α-hel injected male animal, it was lower than NoRS control male group, whereas both male and female stressed α-hel injected mice had lower Exploration Index than the RS control male group.

An effort was made to assess the effect of estrous cycle phase on anxiety-like behavior and cognitive functioning. Several findings in the literature suggest that estrous cycle is able to affect the cognitive and behavioral outcomes of the female mice. For instance, Cole et al. (2016) have shown differential cognitive performance of female rodents with respect to the estrous cycle phase; mice in diestrous had similar sustained attention performance as male rats, while rats in the proestrous or estrous phases performance better than the male rats. Furthermore, the higher glucocorticoid levels in females compared to males that is apparent following a stressful stimulus has shown to be affected by estrous cycle (Seale et al., 2004). In contrast, in their study Conrad et al., (2004) showed that acute stress similarly affects

the facilitation of spatial memory in female rats independently of the estrous cycle phase. In this study, the performance of a subgroup of female mice in the Light-Dark and TOR tasks were assessed in respect to each animal's estrous cycle phase. The inconsistence of the results and the limited number of subjects rendered it difficult to reveal a correlation between the estrous cycle phase and the performance of animals.

7. CONCLUSIONS

Based on this study, certain conclusions can be drawn about the effects of acute restraint stress on the anxiety and mPFC related cognitive functions of male and female mice. Stressed female mice spent significantly less time in the dark compartment of the Light-Dark apparatus compared to the unstressed mice, an effect of higher anxiety level in the female and not male RS group. However, the Discrimination Index of the male RS mice was significantly lower than that of the NoRS male mice, while the DI of NoRS and RS female mice was comparable. These effects suggest restraint stress impairs recency memory in male but not female mice. The anxiety levels and cognitive function of the male control NoRS and cannula-implanted mice were not significantly different, however more animals are needed for the characterization of cannula implantation implications.

Regarding the α-helical CRF (9-41) effects, both NoRS and RS male α-helical injected mice showed similar Discrimination Index in the Temporal Object Recognition task, an effect indicative of the restraint stress-induced cognitive impairment by α-helical CRF (9-41) injection. In the Light-Dark test the female α-helical injected RS mouse spent less time in the dark compartment compared to the female control RS group. Finally, no effect of estrous cycle was found on anxiety and cognitive functioning of the female mice. Future studies should elucidate the effects of α-helical CRF (9-41) on anxiety and stress-induced cognitive dysfunctions gathering data from a greater number of experimental animals.

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