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Comorbidity of alcohol use disorders (AUDs) and anxiety disorders: molecular mechanisms and the role of psychosocial stress in a rat model (*Rattus norvegicus*)

Andrea Coppola

Examiner, Urban Friberg
Supervisor, Carlos Guerrero-Bosagna

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Författare
Author

Andrea Coppola

Sammanfattning
Abstract

Alcohol use disorders (AUDs) are psychiatric disorders characterised by impaired control over alcohol intake, compulsive use (use of alcohol despite its aversive effects) and increased negative affective state associated to withdrawal. Epidemiological studies report that AUDs are highly comorbid with anxiety disorders in humans. The involvement of dysregulated stress systems in the pathophysiology of alcohol use disorders consolidates the idea of neurobiological overlaps between AUDs and anxiety disorders. Indeed, repeated stressful experiences can both escalate alcohol dependence and induce uncontrollable anxiety levels. Given that psychosocial stress is one of the main stressor humans are exposed to during their lifetime, increasing interest in the field is focusing on the effects of secondary trauma, or the direct witnessing experience of somebody else's pain. However, despite the recent progress in this field, overlapping molecular mechanisms between AUDs and anxiety disorders are not fully understood. Additionally, sex-specific effects of psychosocial stress are understudied due to the lack of proper animal models. In one study, we investigate the role of PRDM2, a gene encoding for a histone methyltransferase previously reported to play a role in alcohol use escalation, in a model for posttraumatic stress disorder (PTSD) in rats (*Rattus norvegicus*). We show that downregulation of PRDM2 in the prelimbic cortex increased fear expression in cued fear conditioning paradigm. In a second study, we report on the potential of a foot shock/witness paradigm in rats to bridge the gap between physical and psychosocial stress and that can be used as a model for sex-dependent effects.

Nyckelord
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alcohol use disorders, anxiety, comorbidity, epigenetic mechanisms, PRDM2, social stress, witness.

Contents

1 Abstract

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Keywords: alcohol use disorders, anxiety, comorbidity, epigenetic mechanisms, PRDM2, social stress, witness.

2 Introduction

2.1 Alcohol use disorders and anxiety disorders are highly comorbid

Alcohol use disorders (AUDs) are psychiatric disorders characterised by impaired control over alcohol intake, compulsive use (use of alcohol despite its aversive effects) and increased negative affective state associated to withdrawal (Hasin et al., 2012). Globally in 2016, alcohol use disorders accounted for 0.3 % of all the deaths and had a prevalence of 5.1 % among the population aged 15+ years (World Health Organization, 2018). AUDs and anxiety disorders are highly comorbid in humans, with 37 % of alcohol dependent patients who were diagnosed for anxiety disorders during the previous year (Kessler et al., 1996). Above all, patients with posttraumatic stress disorder (PTSD) are more likely to develop AUDs than people without PTSD (Jacobsen et al., 2001). Indeed, the rewarding effect of acute alcohol exposure might represent a self-medication to ameliorate the negative affective states in anxiety disorders (Markou et al., 1998; Logrip et al., 2012). Neurobiological overlaps between the two psychiatric disorders, support this idea (Markou et al., 1998). However, the molecular mechanisms underlying this comorbid phenotype, are still not fully understood. We will briefly discuss the achievements of the field in this respect.

2.2 Neurobiological basis for comorbidity and molecular mechanisms

Since the early 1990s, researchers have tried to identify a neurobiological basis behind comorbid AUDs and anxiety disorders. Evidence in the field supports this bidirectional relationship, showing how sensitivity to stress exposure and negative affective states is central to alcohol addiction pathology (Koob et al., 2014). George Koob and colleagues (2014) suggest that transition from alcohol use to alcohol abuse comes from dysregulations of the stress systems. The initial rewarding effects of acute alcohol that motivates alcohol seeking (positive reinforcement) are then replaced by the motivation to decrease affective state, resulting from withdrawal, associated to impaired stress and mood systems (negative reinforcement) (Koob et al., 2014). Moreover, increasing evidence suggests neurobiological overlaps between alcohol use and anxiety disorders due to similar anatomical regions and projecting areas, cellular mechanisms and molecular modifications being involved (Markou et al., 1998; Breese et al., 2011).

Several studies in the past decade focused on the role of medial prefrontal cortex (mPFC) in alcohol use disorders in rodent models (Moorman et al., 2015). mPFC is an element of the mesocorticolimbic system involved in drug-related behaviour (Kalivas, 2008) which has been associated with fear-related and anxiety-like behaviour (Maren & Quirk, 2004). mPFC is a multifaceted brain region with different subdivisions showing distinct anatomical and functional characteristics (Heidbreder & Groenewegen, 2003). mPFC is divided into a dorsal mPFC, which includes pre-central cortex (PrC) and anterior cingulate cortex (ACC), and a ventral mPFC, which includes prelimbic (PL), infralimbic (IL) and ventral orbital (VO) cortices (Heidbreder & Groenewegen, 2003). Special interest has been spent over the functional distinction of PL and IL cortices, which are involved in emotional and motivational processes (Vertes, 2004). A simple model suggested that PL mostly promotes the motivational seeking of drugs and natural rewards whereas IL inhibits it ("go" vs "no-go" model) (Moorman et al., 2015). However, recent findings provided a challenge to the simple *go vs no-go* model, as more complex interactions between mPFC circuits are recruited in alcohol seeking behaviour and fear conditioning (Willcocks & McNally, 2013; Pfarr et al., 2015). For instance, evidence shows that the IL inhibitory control of the behavioural output is not generalised, with distinct neuronal ensembles within this region working in a synergic way (Pfarr et al., 2015). Moreover, Burgos-Robles et al. (2009) reported that PL, in addition to its role in promoting fear expression, it is also involved in extinction mechanisms.

mPFC projects to other reward-related systems, such as the nucleus accumbens (NAc) and receives inputs from both the sensorimotor and limbic systems (Kalivas, 2009). Above all, several studies reported that mPFC-to-amygdala projections are involved in both fear conditioning and alcohol seeking behaviour, supporting the idea that dysregulations in these projecting areas might contribute to comorbid phenotypes (Peters et al., 2009; Gilpin & Weiner, 2017). In models of PTSD, prior exposure to traumatic stress increased excessive drinking and altered PFC-to-amygdala activity (Edwards et al., 2013). Specifically, altered balance of prefrontal cortex activity can cause hyperactivity of amygdala nuclei, mainly the basolateral amygdala (BLA), whose dysregulation is associated to both anxiety and addiction (Tye et al., 2011; Rau et al., 2015; Sharp, 2017).

At a cellular level, the hyperactivity of amygdala nuclei can be due to the modulation of GABAergic transmission (Prager et al., 2016). Several neuronal peptides have been reported to directly regulate anxiety and alcohol dependence via modulation of GABAergic circuitry, with

corticotropin releasing factor 1 (CRF1) and neuropeptide Y (NPY) exerting opposite effects (Gilpin et al., 2015). CRF1 has been shown to increase withdrawal-induced anxiety-like behaviour and alcohol drinking, whereas intra-amygdala NPY injection reduces these effects (Overstreet et al, 2003; Gilpin et al., 2011).

At a molecular level, epigenetic modifications are good candidates to study the bidirectional relationship between alcohol use disorders and anxiety disorders, since both chronic ethanol exposure and chronic stress induce long-term neuroadaptations. Epigenetic mechanisms are mechanisms that specifically change chromatin structure rather than DNA sequences, leading to differential gene expression (Allis et al., 2007). Intense work has shown the role of DNA methylation and histone acetylation processes in regulating brain-derived neurotrophic factor (BDNF) which is involved in synaptic plasticity and whose dysregulation is associated with the pathophysiology of alcohol dependence and anxiety disorders (Moonat & Pandey, 2012). Moreover, inhibition of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) prevented the escalation of alcohol intake in a rat model (*Rattus norvegicus*) (Warnault et al., 2013). However, little is known about other epigenetic modifications, such as histone methylation. A previous work showed that the expression of a histone methyltransferase, PR domain containing 2 (PRDM2), was significantly decreased in post dependent rats (Barbier et al., 2016). PRDM2 is a tumor suppressor enzyme whose inactivation has been studied in relation to human cancers (Poetsch et al., 2002). This enzyme is significantly expressed in the human prefrontal cortex suggesting an important role in this brain region (http://biogps.org). Viral-induced downregulation of PRDM2 in the dmPFC was able to mimic the behavioural phenotype of alcohol dependence, such as increased alcohol intake and aversion-resistant consumption (quinine adulteration) (Barbier et al., 2016).

However, the role of PRDM2 has never been investigated in other psychiatric disorders. Therefore, the focus of our first study (Experiment 1) was to evaluate the effects of PRDM2 downregulation in PL cortex in fear behaviour, using a cued fear conditioning paradigm in rats (*Rattus norvegicus*) as a model for posttraumatic stress disorder (PTSD). Moreover, we investigated the potential involvement of specific afferent projections from the PL cortex, focusing on the role of basolateral amygdala (BLA), involved in emotional and motivational processes, and periaqueductal grey (PAG), involved in fear and pain processing. We hypothesised that PRDM2 knock down would result in increased fear expression in cued fear

conditioning paradigm, consolidating the hypothesis of neurological and molecular overlaps between alcohol dependence and anxiety disorders.

2.3 Behavioural models for comorbidity: the role of psychosocial stress

Evaluating overlapping mechanisms between AUDs and anxiety disorders helps the clinical research to find possible pharmacological targets for treatments. However, the effectiveness of the treatments is strictly correlated to the validity and the predictive value of behavioural models used in the lab (Yardley & Ray, 2017). Indeed, researchers sought to model some clinical aspects of comorbid phenotype of AUDs and anxiety disorders in animal models, especially rodents, to predict possible hallmarks and major risk factors for the human condition, but most of them have limited translational value (Spanagel, 2017). Above all, basic research focused on the role of stress exposure and consequent anxiogenic symptoms to enhance misuse of alcohol, as a consolidation for the self-medication theory (Gilpin & Weiner, 2017). Despite the difficulties to produce animal models for long-lasting effects of stress exposure in alcohol drinking (Boyce-Rustay et al., 2008), the field has been more successful in modelling how traumatic stress can escalate prior alcohol drinkers or reinstate an extinguished alcohol dependence (Mantsch et al., 2016). The role of different types of stressors have been examined (Noori et al., 2014). Daily forced swim stress accelerates the onset of alcohol dependence in adult male C57BL/6J mice (*Mus musculus*) (Anderson et al., 2016) and repeated exposure to foot shocks increases voluntary alcohol intake in a two-bottle choice home cage alcohol drinking in alcohol-naïve rats (Meyer et al., 2013). Edwards et al. (2013) reported that rats with high sensitivity to bobcat urine odour showed higher alcohol drinking over three weeks post-stress and more aversion-resistance to taste adulteration than low sensitive rats.

Researchers have shown special interest for the role of social stress, since it represents most of the stress humans are exposed to during their lifetime (Almeida, 2005). Rats are valuable animal models for social stressors since they are very social animals, as they live in large colonies in the wild (McEwen et al., 2015). Several studies used a model of social isolation during adolescence and social defeat stress (Gilpin & Weiner, 2017). Early studies showed that adolescent social isolation increased alcohol consumption (Wolffgramm et al., 1990). Moreover, Long Evans rats that were socially isolated starting from postnatal day (PD) 28, showed increased anxiety-like behaviour in an elevated plus-maze (EPM) and hyperactivity in a novel environment (Chappell et al., 2013). Low-drinking rats exposed to ten episodes of social

defeat stress showed higher alcohol intakes in operant self-administration than unstressed controls, after that their cue-guided alcohol seeking was extinguished (Logrip & Zorrilla, 2012).

Even though most of social stressors imply physical interactions, merely psychological components play an important role in stress-induced consequences (Nicodimos et al., 2009; Feinstein et al., 2014). In humans, witnessing parental violence or other traumatic events (e.g. war, crime) increases the vulnerability to develop anxiety disorders and depression-like symptoms (Nicodimos et al., 2009). Recent evidence shows that physical and psychological social stress lead to different physiological and behavioural outcomes (Finnell et al., 2017). Animal studies show the negative effects of this secondary trauma, also referred to as vicarious stress (Church, 1959; Zalaquett & Thiessen, 1991; Atsak et al., 2011). Rats show vicarious freezing when witnessing cage mates experiencing repeated foot shocks (Atsak et al., 2011) or aversive odours (Zalaquett & Thiessen, 1991). However, few studies showed the effects of vicarious stress on anxiety-like behaviour and other depression-like symptoms. Warren et al. (2013) used a social defeat/witness paradigm in mice to report enhanced anxiety-like behaviour after witnessing other mice experiencing defeat episodes. Patki et al. (2014) showed similar effects of witnessing social defeat in rats. They showed, both in defeated and witnessing rats, that anxiety-like behaviour was significantly higher when the rats were single housed following the social defeat/witness paradigm than when they were let interact in between stress episodes.

Despite its ethological relevance, one caveat of social defeat paradigm is that it does not allow direct comparison between the sexes, since in many rodent species females do not display spontaneous aggression towards conspecifics (Haller et al., 1999). Few animal models exist to test the effects of social stress in females, and if present, they do not provide a valid resource for direct comparison between the sexes (Finnell et al., 2017). Developing such a model is crucial given that epidemiological studies from the human population report higher sensitivity of females to stress (Brougham et al., 2009) and higher susceptibility to depression-like behaviours than males (Hankin et al., 1998).

For this reason, in our second study (Experiment 2) we developed a behavioural model in rats to investigate the role of psychosocial stress and its sex-specific effects in anxiety-like behaviour and comorbidity with alcohol use disorders (AUDs). The model consists of exposing one individual to repeated foot shocks while a cage mate is forced to witness the traumatic experience. We will present the results from a pilot study carried out with male rats,

hypothesising that witnessing a cage mate exposed to repeated foot shocks would elicit vicarious stress and would result in (1) increased anxiety-like behaviour in an elevated plus-maze and (2) open field and (3) increased social avoidance towards unknown conspecifics in a social interaction test.

3. Materials and methods

3.1 General methods

3.1.1 Animals and housing conditions

Male Wistar rats (*Rattus norvegicus*) were the subjects for all the studies reported here. The animals were housed in individual-ventilated cages (IVC, 51 cm x 35 cm x 27 cm) (Allentown Europe LTD, Reading, England) in groups of four at the Centrum för biomedinska resurser (CBR) at Linköping University, Campus US. After their arrival, all the animals were left habituate to the facility for one week prior to any experimental procedure. Group size and composition was modified according to the specific experimental plans (See sections 3.2.1.1 and 3.2.2.1). An automated system (ecoflo, Allentown Europe LTD, Reading, England) controlled the temperature and relative humidity conditions of the cages, keeping standard values of 22-23 °C and 66-68 % respectively. The enrichment of the cages consisted of chopped-wood bedding, nesting material (e.g. straw), cardboard rolls and wooden sticks to let the animals chew and avoid teeth damaging (Figure 1). Food and water were provided *ad libitum*. Animals were housed at a fixed 12 h/12 h dark/light cycle, with lights turning off at 07:00 and turning on at 19:00. Given that rats are nocturnal animals, all experimental procedures were carried out during the dark phase (07:00-19:00) when the animals show most of their active behavioural repertoire (Refinetti, 2004). We performed all the routine work under red lights to never expose the rats to white light during the dark phase and to not disrupt their circadian rhythms.

Figure 1: Typical enrichment for rats' IVC cages.

3.1.2 Handling

One week after their arrival in the facility, the animals were handled daily by the same experimenter in order to avoid unwanted stressful experiences during the testing. Handling procedures consisted of picking up the animals from their home cage, letting them sniff the gloved hands and the arms of the experimenter for 2-3 minutes. Moreover, animals were lifted a couple of times, mimicking the action of moving them from a place to another. Handling procedures included tail marking and weighting.

3.1.3 Anxiety-like behaviour tests

We tested anxiety-like behaviour in rats using validated behavioural assays, such as the elevated plus maze (EPM) and the open field test (OF). To avoid unwanted stress due to novel contexts, we let the animals habituate to the room where the behavioural tests was carried out for at least one hour before the test. We moved the animals within their home cages to the experimental room and we removed the filters from the cages to let the animals familiarise with the room's odours for at least one hour. We used only artificial red light to brighten the room, to avoid exposing the rats to white light.

3.1.3.1 Elevated Plus Maze (EPM)

The maze consisted of two open arms (51 cm long), two closed arms (51 cm long) bordered by 41 cm high walls and a central neutral square (10 cm x 10 cm). The maze was extending 73 cm above the floor. We placed the maze in the room so to avoid any shadowing on the arms and to avoid biased results, given rats' preference for darker places. We placed the maze so to expose the open arms to the widest open area of the room and the closed arms facing the walls of the room (Figure 2). The procedure followed previous validated methods (Pellow et al., 1985).

Figure 2: EPM set up. 1. Video camera; 2. Red light; 3. Maze.

We moved one subject per time from its home cage to an empty cage, deprived of any enrichment, except for the bedding. During the transport from the animal room to the testing room, the cage was covered with a dark cloth to avoid exposing the animals to direct white lights in the corridor. Once in the testing room, rats were lifted and placed on the EPM facing one of the closed arms. The experimenter left the room immediately after laying the animal on the maze. We let the animals explore the maze for 5 minutes before picking them up and returning them to their home cage. We cleaned the maze in between each test and before starting the first one using a 70 % ethanol solution, to avoid confounding due to olfactory cues. We videotaped all the tests and we scored the videos manually using a stopwatch. We scored the total time spent and total entries in the open arms and closed arms. We considered as a full entry when the rats crossed the central square with all the four paws (Figures 3a,3b) and we stopped the watch as soon as the rats' head was out of the arm back to the central square (Figure 3c). When the animal put its head out of the arm back to the central square, but not fully exited the

arm, we started the watch again as soon as the animal was returning back to the arm, but we did not consider that as an entry (Figure 3d).

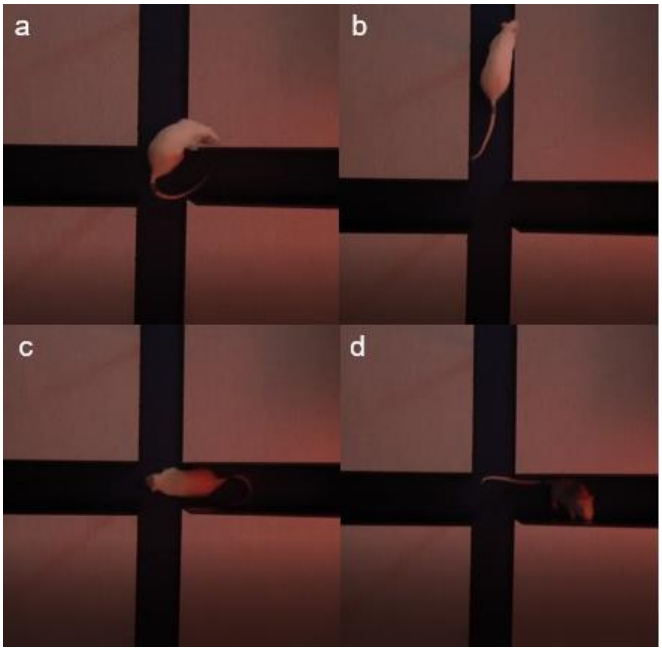

Figure 3: EPM Scoring examples. a. Subject stretching out of the arms but no entry is scored; b. Example of complete entry. c. Subject heads out of the closed arm and (d) back again.

3.1.3.2 Open Field Test

The room and light conditions were the same as described for Section 3.1.3. The procedure followed previous validated methods (Seibenhener, & Wooten, 2015). The animals were moved one per time from their home cage to an empty cage, deprived of any enrichment, except for the bedding. During the transport from the animal room to the testing room, the cage was covered with a dark cloth to avoid exposing the animals to direct white lights in the corridor. Once in the testing room, rats were lifted and placed in the centre of an open field arena (75 cm x 75 cm x 50 cm). The experimenter left the room immediately after laying the animal in the arena. We let the animals explore the arena for 20 minutes before picking them up and returning them to their home cage. We cleaned the arena in between each test and before starting the first one using a 70 % ethanol solution, to avoid confounding due to olfactory cues. We videotaped all the tests and we scored the videos using a video tracking software, Ethovision (Noldus, Amsterdam, The Netherlands). We scored total distance moved and time spent in the centre of the arena (45 cm x 45 cm) in five minutes bins, using centre-point tracking system.

3.1.4 Anaesthesia and sacrifice

After the end of the experimental plan, we euthanized all the animals by decapitation. We strongly sedated the animals by inhalation of isoflurane (Baxter, Deerfield, Illinois) in a box (41 cm x 20 cm x 25 cm) with oxygen in-flow, together with two to three cage mates. We tested successful anaesthesia by checking for absence of sensorial response to external stimuli (e.g. sudden noise) and absence of nociception (we gently pinched one of the paws to check for toe pinch withdrawal reflex). If anaesthesia was confirmed, we quickly moved the rats to a guillotine and we decapitated them with a quick and firm move to prevent them from waking up and to avoid unwanted suffering.

3.2 Experiment-specific methods

3.2.1 Experiment 1: PRDM2 Knock Down

3.2.1.1 Animals

In order to test the hypothesis that PRDM2 knock down in the prelimbic (PL) cortex affects fear-related behaviour, we used a first batch of animals (n = 40) to test its effect in cued fear expression 24 hours after the acquisition of fear. To test the hypothesis that the PRDM2 knock down effects were not time-dependent, we used a second batch of animals (n = 24) to test these effects in cued fear expression one week after the acquisition of fear. Finally, to test the hypothesis of specific projecting areas involved in the PRDM2 knock down effects, we used a third batch (n = 80) to test the involvement of the projecting neurons from the PL cortex to the basolateral amygdala (BLA) and periaqueductal grey (PAG) in cued fear expression 24 hours after the acquisition of fear. Moreover, we used the animals from the first and the third batch to control for any effect of the virus-induced knock down in baseline anxiety-like behaviour using EPM and the Open Field test. All the animals arrived at the facility at postnatal day PND 70 and were housed in IVC by groups of four.

3.2.1.2 Experimental plan

Experimental plan is schematized in Figure 4. Upon their arrival at the facility, we let the animals familiarise with the new housing conditions for one week prior to any experimental manipulation. The following week, we handled, marked and weighted the animals to let them habituate to the experimenter and to common procedures. After one week the animals

underwent surgery where we injected a virus bilaterally in the desired brain regions (See section 3.2.1.3). Then, we waited four weeks for the incubation of the virus to allow successful knock down of the gene of interest. Then, the animals underwent cued fear conditioning paradigm as described in section 3.2.1.4. We ran control behavioural tests (EPM and OF) before the fear conditioning paradigm to determine baseline anxiety levels. After all the tests were run, we sacrificed the animals following the procedure described above (Section 3.1.4).

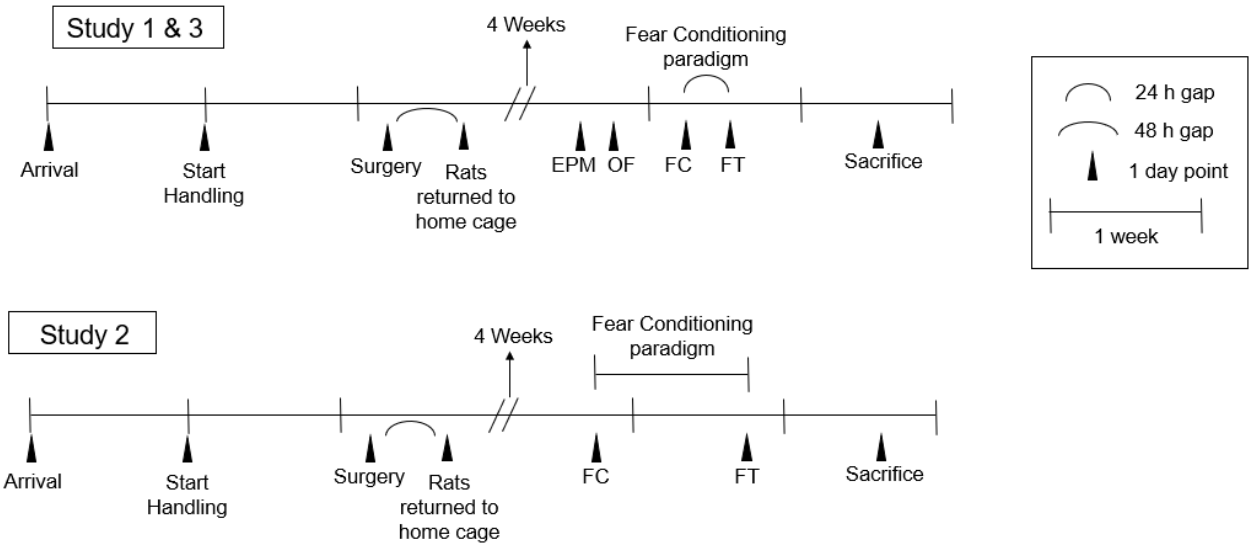

Figure 4: Experimental plan for Experiment 1. EPM = Elevated Plus Maze; OF = Open Field; FC = Fear conditioning; FT = Fear Test.

3.2.1.3 Stereotactic surgery

The animals were anesthetized by inhalation of isoflurane (Baxter, Deerfield, Illinois). Successful anaesthesia was monitored by gently pinching one of the paws to rule out any pain sensation (toe pinch withdrawal reflex). We then injected subcutaneously buprenorphine (Temgesic ©) (0.1 mg/kg) for pain relief. The animal was then moved to a stereotactic apparatus (for detailed protocol of surgical procedure, see Cetin et al., 2006) and the virus was injected bilaterally to the desired brain regions.

For the first and the third batch (n = 40;n = 24) , one group of animals (n = 20; n = 12) received injection of a recombinant adeno-associated virus 9 (rAAV9) carrying a short hairpin RNA (shRNA) for PRDM2 gene knock down in the PL cortex, and another group (n = 20; n =12) served as scrambled control (Figure 5a). Once expressed, the shRNA is processed by the

Dicer/RISC complex machinery to target the degradation of PRDM2 mRNA in the cytoplasm, decreasing its expression level. In the second batch, we divided the animals in two groups, one targeting the BLA (n = 40) and the other targeting the PAG (n = 40). In both the groups, one half (n=20) served as scrambled control and the other (n = 20) received bilateral injection of a rAAV9 in PL cortex. However, this time the rAAV9 vector was designed to express the shRNA in a Cre-dependent mechanism (DIO cassette, Saunders & Sabatini, 2015): so, the animals received an additional bilateral injection of a rAAV2-retro-Cre virus either in the BLA or in the PAG. This virus can retrogradely infect cells projecting to the BLA or the PAG such as cells that project from the Pl to the BLA or from the PL to the PAG (Figure 5b). Once in the PL, the rAAV2-retro-Cre was able to express a carrying Cre recombinase gene, therefore activating the expression of the shRNA targeting PRDM2 and resulting in its downregulation. By this, PRDM2 knock down in the prelimbic cortex was selective in the projecting neurons to either BLA or PAG.

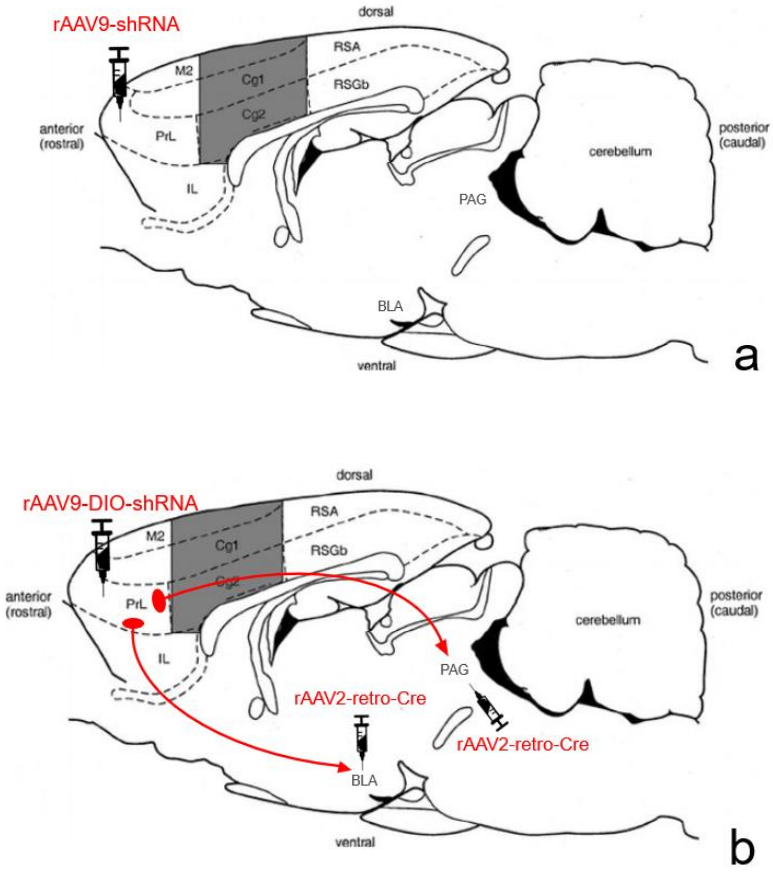

Figure 5: Schematic representation of virus-induced PRDM2 knock down in the PL cortex: AAV9.HI.shR.Prdm2.CMV.ZsGreen.SV40 (a); conditional gene knock down in PL cortex through retrogradely virus expression AAV-retro2-hSyn1-EGFP_iCre-WPRE-hGHp(A) (b). Figure adapted from Cardinal et al. (2003).

After the virus injection, we sewed the wound and let the animals fully recover monitoring their body temperature; after recovery we injected an analgesic, ketoprofen (Rifen © 0,1 mg/kg) before placing them in a small cage (30 cm x 32 cm x 19 cm). We pair housed all the animals after surgery to prevent signs of distress. We monitored the animals to look for their recovery and we injected ketoprofen subcutaneously for additional two days before returning the rats to their home cage with their original cage mates (groups of four).

3.2.1.4 Cued fear conditioning paradigm

Cued fear conditioning paradigm follows the principle of Pavlovian classical conditioning. The animals learned to associate a neutral innocuous stimulus (e.g. a tone, Conditioned Stimulus, CS) to a relevant aversive stimulus (Unconditioned Stimulus, US), in our case a foot shock. Further exposure to CS made the animals react as they would react in response to US; the new learned response is referred as conditional response (CR). After the acquisition of fear, we tested fear expression by exposing the animals to the CS alone, without US and we scored the extent of CR, expressed as freezing behaviour. We scored freezing as the only parameter for fear expression since it is a natural antipredator behaviour and it is sensitive to low levels of fear (Blanchard & Blanchard, 1969). The procedure followed previous validated methods (Maren, 2001). The test consisted of two days. The first day was the conditioning phase. We placed the animals in an operant chamber (25 cm x 32 cm x 26 cm, Med Associates Inc, Saint Albans, Vermont) furnished with a house light, a speaker for acoustic stimulation, a grid floor (nineteen steel grids, 0.5 cm diameter, 1.5 cm interspace) for induction of foot shocks and a camera for video tracking (Figure 6).

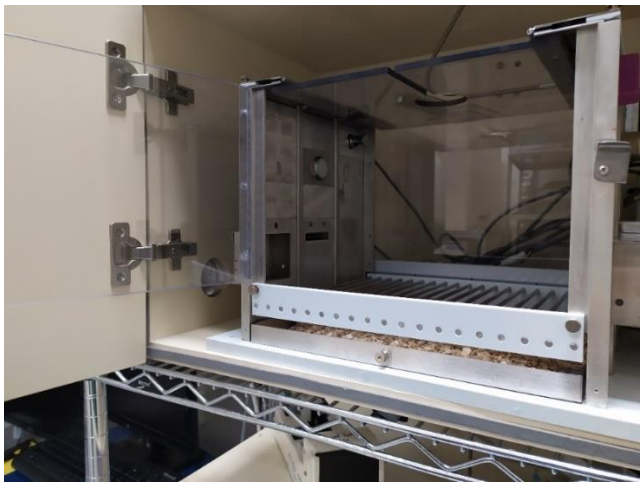

Figure 6: Operant chambers for fear conditioning paradigm.

All the chambers were controlled by a computer software Med-PC IV (Med Associates Inc, Saint Albans, Vermont) that set all the conditions of the trial. When the program started, the house light was switched on and the camera started recording. The animals were let habituate to the chamber for five minutes. After that, a tone (0.75 dB), the CS, was delivered for 30 seconds, with a foot shock applied in the last 2 seconds (0.8 mA). We exposed the animals to 6 consecutive tones/foot shocks with an interval of 3 minutes between each tone/foot shock. After the last tone, the animals spent an extra minute in the chamber before the light went off and after that, we returned them to their home cage (Figure 7a). The second day was the testing phase. In this phase, we let the rats habituate for 5 minutes before the first tone (0.75 dB) was delivered for 30 seconds, followed by no foot shock. A total of six tones was delivered with an interval of 3 minutes between each tone. After the last tone, the light went off and we returned the animals to their home cage (Figure 7b).

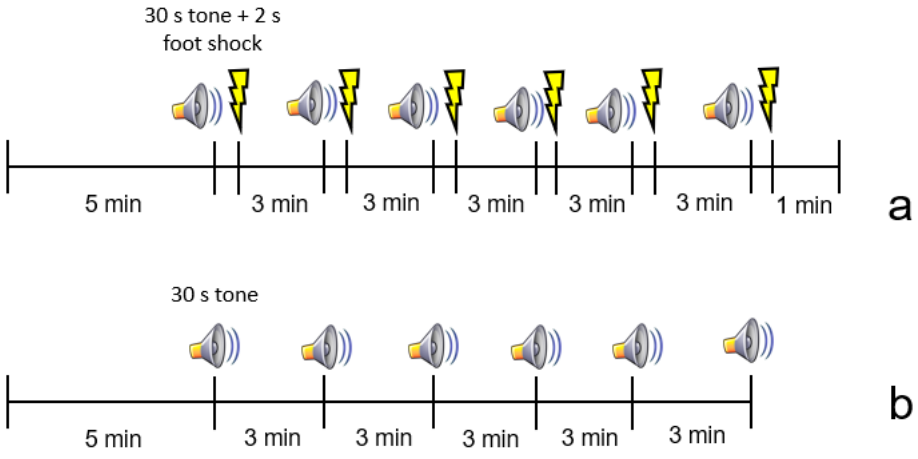

Figure 7: Schematic drawing for fear conditioning (a) and fear test (b).

Videos were downloaded and freezing behaviour was scored manually both in the conditioning and testing phases. We defined freezing as a complete absence of movement except for breathing (Blanchard & Blanchard, 1969). We expressed freezing behaviour as percentage of total time spent freezing over the time of each tone duration. We used the average freezing between the first two tones in the testing phase as a parameter for fear expression, given that the first two tones are more indicative of fear acquisition (Myers & Davis, 2006).

3.2.2 Experiment 2: Foot shock/witness paradigm

3.2.2.1 Animals

We used one batch of twenty animals to test the effectiveness of a foot shock/witness stress paradigm. All the animals arrived at the facility at PND 42 and were housed in IVC by groups of four.

3.2.2.2 Experimental plan

Experimental plan is schematized in Figure 8. Upon their arrival to the facility, we let the animals habituate to the new housing for at least one week prior to any experimental procedure. After one week, we started handling the animals to make them habituate to the experimenter and to experimental manipulation. One week later, we ran an open field test and a social interaction test to score the baseline levels of locomotion and social interaction with an unknown conspecific (See section 3.2.2.4): this was to avoid random assortment of the animals in the experimental groups and prevent from biased results in the following behavioural assays. We then assigned each animal to any of the three experimental groups keeping even average values for locomotion and social interaction for all the groups. The groups were the following: Stress (n = 7); Witness Stress (n = 7); Control (n = 6). Once assigned to each group, we pair housed the animals in smaller IVC cages (30 cm x 32 cm x 27 cm, Allentown Europe LTD, Reading, England) keeping the same enrichment and temperature and humidity conditions. Each rat belonging to the stress group was housed with the one that would have witnessed its foot shock stress exposure; each control rat was housed. with another control rat. Then, we let the animals rest for one week; in this week we collected blood samples from the tail vein for baseline corticosterone levels, as described in Section 3.2.2.5. After one week of rest, we started with the foot shock/witness stress paradigm (See section 3.2.2.3) when we exposed the rats to one session a day per five consecutive days. Immediately after the first session, we single housed the animals in small IVC cages (30 cm x 32 cm x 27 cm), given that social housing can buffer the stressful experiences (Patki et al., 2014). We also deprived the Stress and Witness Stress animals of the rolls, given that this enrichment can alleviate the post-stress symptoms (Lehmann, & Herkenham, 2011). We single housed controls as well but we did not remove the rolls from the cage, given that removal of enrichment on itself might precipitate anxiety-like behaviour (Smith et al., 2017). Moreover, to avoid unwanted stressful experiences due to social separation, we let them interact with their former cage mates for at least one hour every day and prior to any behavioural test. After the first session and the last one, we collected blood samples

from the tail vein from each animal, nearly ten minutes after we removed the animal from the operant chamber, given that the stress response is revealed in elevated plasma corticosterone and peak out after five minutes (Thanos et al., 2009). After the foot shock/witness paradigm, we let the animals rest for one week. Exactly one week after the last stress exposure we tested social interaction with an unknown conspecific and the week after we tested for anxiety-like behaviour in EPM and OF tests. The animals were then sacrificed following the methods described in section 3.1.5.

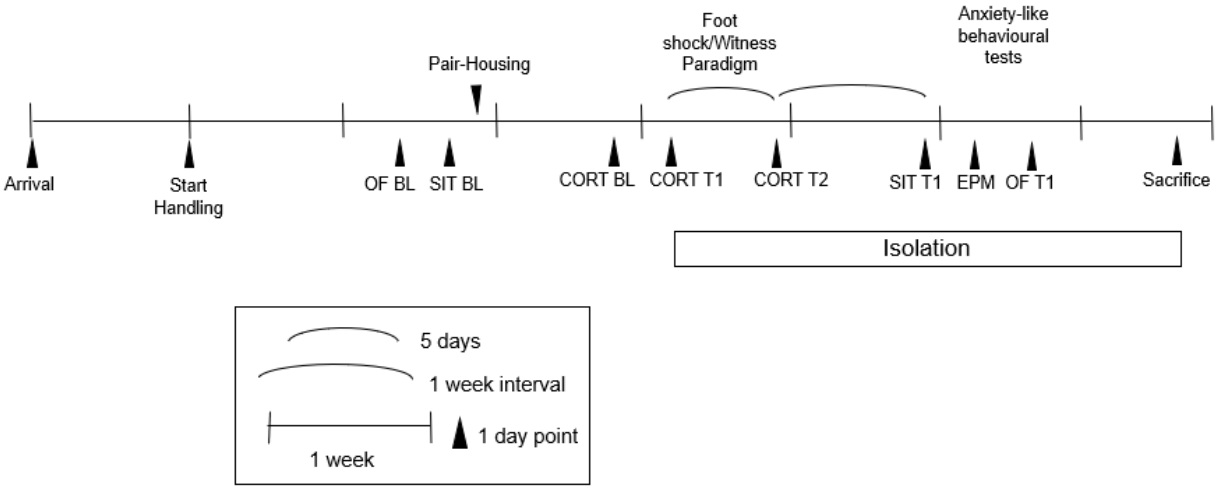

Figure 8: Experimental plan for Experiment 2. BL = Baseline levels; T1 = Time point 1; T2 = Time point 2; OF = Open Field test; SIT = Social Interaction Test; CORT = Blood sampling for corticosterone levels; EPM = Elevated Plus Maze.

3.2.2.3 Foot shock stress/witness paradigm

We placed the animals in operant chambers, like the ones used for cued fear conditioning paradigm (25 cm x 32 cm x 26 cm, Med Associates Inc, Saint Albans, Vermont). However, these chambers were supplemented with a smaller social box (16 cm x 16 cm x 21 cm, Med Associates Inc, Saint Albans, Vermont) with nineteen steel grids (0.3 cm diameter and 1 cm interspace) composing the floor (Figure 9a). A grid opening was present in the interface between the operant chamber and the social box, so to allow interactions between the animals by visual, acoustic and olfactory cues (Figure 9b).

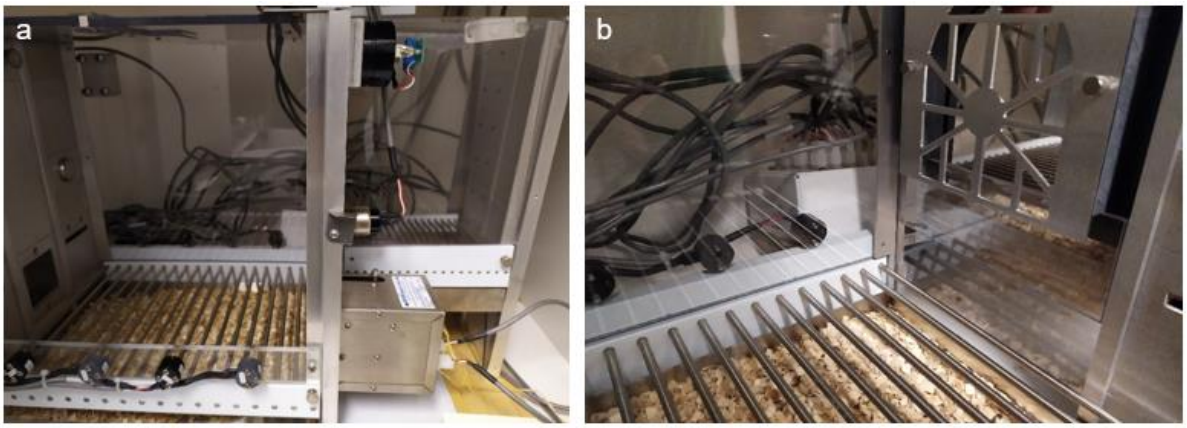

Figure 9: Operant chambers with supplemental social box (a) and grid opening for social interaction between subjects (b).

We used a software, Med-PC IV to control all the parameters for the foot shock/witness paradigm. We placed the stress animals in the operant chamber and the relative witnesses (Former cage mates) in the smaller social box. Once we started the program in Med-PC, the house light turned on and the guillotine door opened to free the grid for social interaction between the animals. We let the animals habituate to the chambers for 1 minute and then a mild tone (0.75 dB) was delivered for 30 seconds; we applied a foot shock (0.8 mA) to the stress animals only in the last 2 seconds of the tone. We exposed the animals to five consecutive tones/foot shocks with an interval of 1 minute between each tone. After the last tone, we let the animals rest for an extra minute before we returned them to their home cage (Figure 10). We treated the controls in the same way, placing them in the operant chamber to control for any stressfulness due to the novel contexts. We did not apply any foot shock to the controls but we still delivered the tones with the same interval of time, to control for any stressfulness due to the acoustic stimulus.

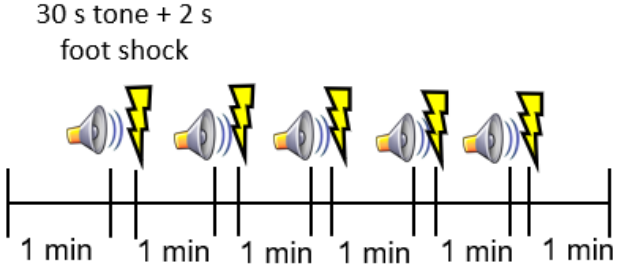

Figure 10: Schematic representation for foot shock/witness paradigm.

3.2.2.4 Social Interaction Test

We carried out a social interaction test at baseline level and one week after the last stress. We used a revised model from the one showed in Lukas et al. (2011). The rationale of this behavioural assay is to test social interaction with an unknown conspecific, given that avoidance of social contact with unknown individuals is a symptom of stress-induced anxiety-like behaviour in rats (Berton et al., 2006). We moved the animals from the animal room to the experimental room one per time, using a small cage (30 cm x 32 cm x 19 cm) with just bedding as described already above (See Section 3.1.3). The test consisted of two phases: in the first phase (Target Absent) we placed the animal in an open field arena (75 cm x 75 cm x 50 cm) furnished with an empty mice wire cage (17 cm x 13 cm x 38 cm) in the middle of one of the arena's walls (Figure 11a). The mice cage was flipped laying on its short size so to avoid the animal from easily climbing on top of it and to maximize the amount of time spent in exploring the cage. We let the animal explore the arena for 10 minutes; after that, we moved to the second phase (Target Present), when we introduced an unknown conspecific within the wired cage: the subjects were able to sniff and see the animal but not direct interaction was allowed. We let the animal explore the arena for extra 10 minutes before we returned both the subjects to their home cages. The social target used for the social interaction test matched the age and the body weight of the test subject, so to maximize interaction. Since the affective state of the social target played a crucial role in the interaction of the test subject, we avoided using only one social target, given that the restraining conditions of the wired cage would have stressed it too much. We always used at least four different social targets per each social interaction test. Additionally, we made sure that each social target met an equal number of times the animals belonging to any of the three experimental groups, to control for the affective state of the social target in the final interaction score average of the groups. We cleaned the maze and the wire caged between each trial and before the very first animal, with a solution 70 % ethanol, to avoid confounding due to olfactory cues. We video-taped all the tests and we scored the videos using a tracking software, Ethovision (Noldus, Amsterdam, The Netherlands). We scored the total amount of time the animal was spending in the Interaction Zone (IZ) in the Target Absent and Target Present phases separately. We defined IZ as a corridor of 9 cm around the wired cage. Furthermore, we scored the time the animal spent in the corners (20 cm x 20 cm) opposite to the wired cage in the Target Absent and Target Present phases separately. We then divided the time spent in the defined regions in the Target Present phase to the time spent in the Target Absent phase [Target Present Time / Target Absent Time] to obtain the interaction score that we used for the analysis. Figure 11b summarizes the relevant zones used for the scoring.

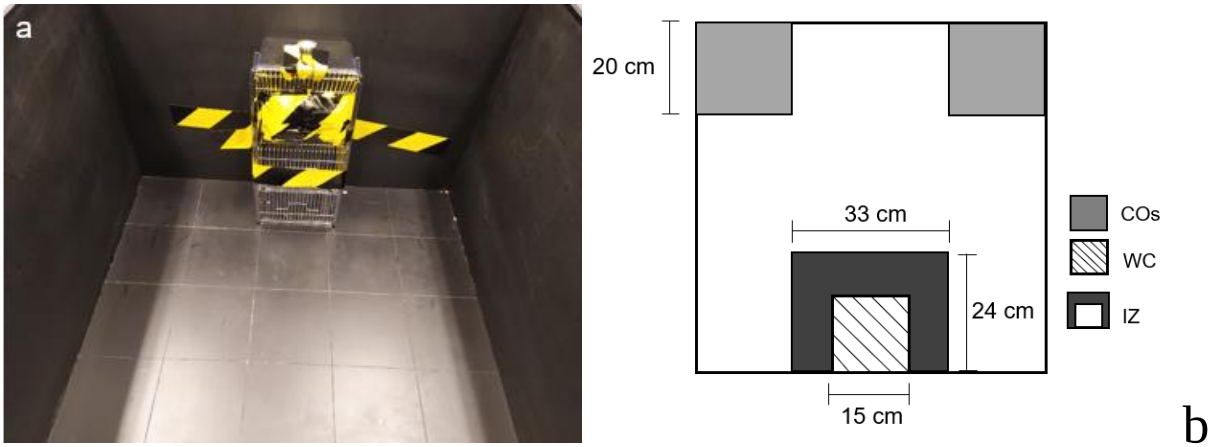

Figure 11: Social Interaction Test set up (a) and schematic representation of its relevant zones for behavioural scores (b). COs = corners; WC = wired cage; IZ = interaction zone.

3.2.2.5 Blood Sampling

We performed blood sampling by collecting blood from the tail vein. We avoided using the standard restraining procedure, given that it is stressful for the animals and this could have biased the results (Kim et al., 2018). Differently, we simply used a cloth to literally wrap the animal in, in order to minimize its struggling during the procedure. With this procedure the animal was still able to move within the cloth, so that it was not restrained, and the darkness of the cloth was useful to calm them down. Once wrapped, we used a 1.5 cm long needle to make a small incision on the tip of the tail vein. Massaging of the tail facilitated bleeding and then we collected blood drops using a heparinised test tube (Microvette ©). We tried to be as efficient and quick as possible to avoid blood clotting and consequently to avoid pinching the animal a second time. We centrifuged the blood samples at 20000 rounds per minute (RPM) per five minutes at room temperature to allow correct plasma separation. We then isolated the plasma from the cellular sediment and we collected it in a LoBind 1.5 ml Eppendorf Tube (Eppendorf International, Hamburg, Germany) and we stored it at -20 °C prior the use.

3.2.2.6 Plasma Corticosterone Analysis

We analysed corticosterone from plasma samples using an enzyme linked immunosorbent assay (ELISA). Before running the test, we processed the plasma collected, in order to clean it up from other relevant biomolecules, that could have interfered with the enzymatic reactions (e.g. lipids). Firstly, we let the samples thaw for at least 30 minutes prior the use. After a quick vortex, we collected 10 µl in a LoBind 1.5 ml Eppendorf Tube, and we added five parts of ethyl

acetate (50 µl). We vortexed the tubes in a ThermoShaker at 1400 rpm for 2 minutes at room temperature and let the organic phases separate for 5 minutes. We then collected the top organic phase and added it to a LoBind 1.5 ml Eppendorf Tube prefilled with 50 µl Milli-Q water. We vortexed the samples in a ThermoShaker at 1400 rpm for 2 minutes at room temperature and let the organic phases separate for 2 minutes. We then collected only the top organic phase in a new empty LoBind 1.5 ml Eppendorf Tube. We repeated the procedure twice to maximize plasma clearance. After that, the plasma samples were ready to be analysed with ELISA assay. If not used immediately, we dried the plasma samples using a vacuum concentrator, SpeedVac (Thermo Fischer Scientific, Waltham, Massachusetts) at low temperature, and we stored the samples at -20 °C. Prior to the use, we resuspended the samples with 50 µl of Assay Buffer (1:5) and then we added 10 µl of it to a LoBind 1.5 ml Eppendorf Tube prefilled with 10 µl of Dissociation Reagent. We let the reagents mix for 5 minutes without shaking and then we added 180 µl of Assay Buffer to each sample to reach the final dilution of 1:100. We then followed the instructions indicated by the kit's manufacturer (Arbor Assay ©, Genprice Inc., San Jose, California) to build the plate. We finally read the optical density generated by each well at 450 nm and calculated corticosterone concentration using a 4-parameters logistic curve calculator using a plate reader.

3.3 Statistical analysis

3.3.1 Experiment 1

We expressed all the data as mean ± SEM. To test the effect of treatments over the percentage of freezing during fear test, we used repeated-measures analysis of variance (ANOVA) adding the different tones as within-subject factors and the treatment as between-subject factor. Then, we ran one-way ANOVA tests of significance to check for effect of treatment over the total distance moved in the open field test, and the relative time spent in the open arms in EPM. For all these tests we checked for the assumption of homogeneity of the variances, running a Levene's test; in case of significance in the Levene's test, we ran the respective non-parametric test for the effect of treatments, instead. We defined significant results when P values were lower than 0.05.

3.3.2 Experiment 2

We express all the data as mean ± SEM. We ran a one-way analysis of variance (ANOVA) to test for any group effect for the relative time spent in the open arms in the EPM, corticosterone level, interaction score and corner score in the social interaction test, and total locomotor activity and relative time spent in the centre in the open field test. For the open field test, we also ran a repeated-measures one-way ANOVA to check for any group effect in the locomotor activity and relative time spent in the centre along the individual five minutes bins. We ran one-way ANOVA separately for the different time points tested. For all these tests we checked for the assumption of homogeneity of the variances, running a Levene's test; in case of significance in the Levene's test, we ran the respective non-parametric test for the effect of treatments, instead. We defined significant results when P values were lower than 0.05. In case of significance, we ran *post hoc* Tukey's test to check for the main group effects.

3.4 Ethical statement

We performed all the experiments under an ethical permit approved by the Local Ethics Committee for Animal Care and Use at Linköping University. We performed all the experiments following 3R (reduce, replace and refine) principle to minimize animal suffering and reduce the number of animals tested.

4 Results

4.1 Experiment 1

4.1.1 PRDM2 Knock Down Prelimbic Cortex

We found that PRDM2 Knock Down (KD) rats showed higher freezing 24 hours after fear conditioning than scrambled rats along the repeated tones (Figure 12, $F_{(1,36)} = 6.068$; $P = 0.019$). Moreover, there was no significant effect of the tones within the subjects, suggesting that the freezing behaviour did not extinguished after the first tones (Figure 12, $F_{(1,36)} = 0.269$; $P = 0.607$).

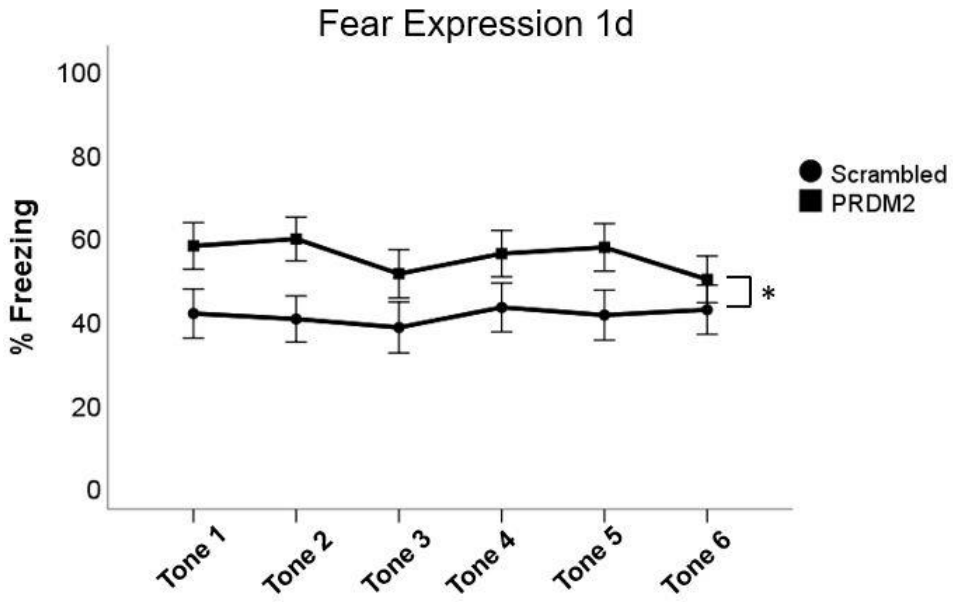

Figure 12: Treatment effect in average freezing along the repeated tones during fear test 24 hours after fear conditioning (Repeated-measures ANOVA, between-subjects effect: $F_{(1,36)}$ = 6.068; P = 0.019). * represents a significant value P < 0.05.

We found that PRDM2 KD treatment did not affect general locomotion of the rats in an open field (Figure 13a, $F_{(1,35)}$ = 0.56; P = 0.459) and did not affect relative time spent in the open arms in EPM (Figure 13b, $F_{(1,36)}$ = 1.564; P = 0.219).

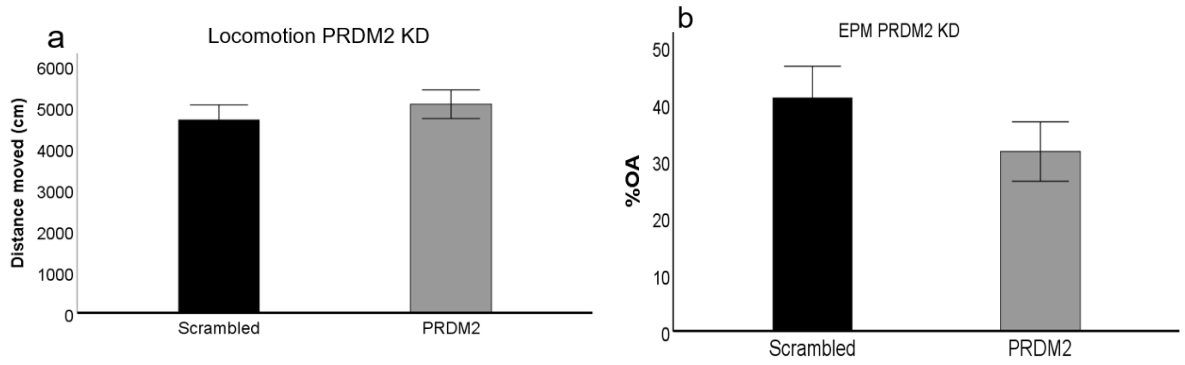

Figure 13: Treatment effect in general locomotor activity (a) and relative time spent in open arms in EPM (b) (One-way ANOVA, locomotion: $F_{(1,35)}$ = 0.56; P = 0.459; EPM: $F_{(1,36)}$ = 1.564; P = 0.219).

We also found that PRDM2 KD rats showed higher freezing than scrambled rats along the different tones one week after fear conditioning (Figure 14, $F_{(1,22)}$ = 5.891; P = 0.024). However,

there was a significant effect of the individual tones within the subjects, suggesting that fear expression was different following the first tones (Figure 14, $F_{(1,22)} = 24.262$; $P < 0.001$).

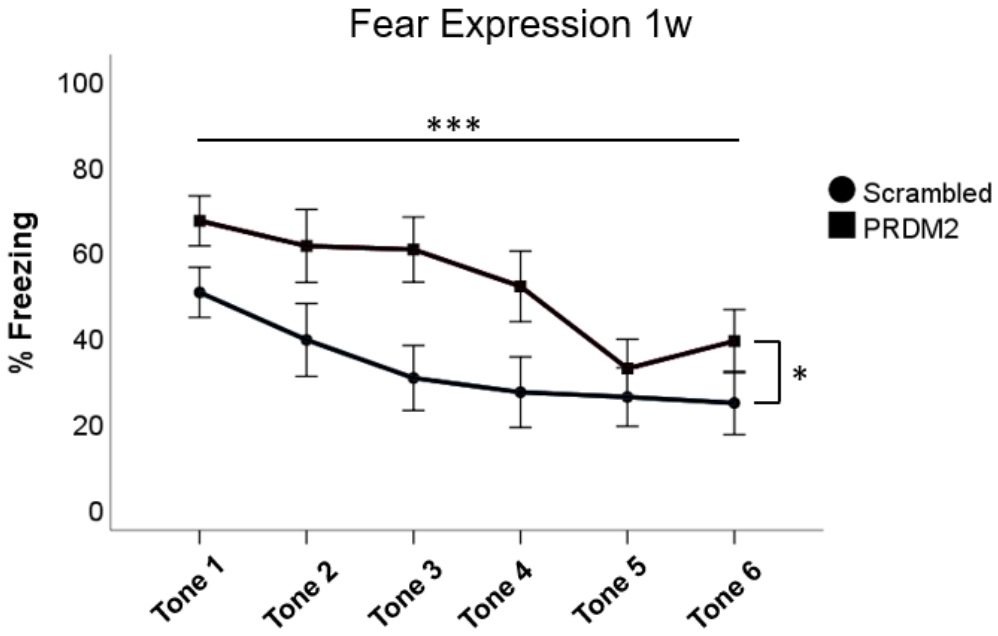

Figure 14: Treatment effect in average freezing along the repeated tones during fear test one week after fear conditioning (Repeated-measures ANOVA; within-subjects effect: $F_{(1,22)} = 24.262$; $P < 0.001$; between-subjects effect: $F_{(1,22)} = 5.891$; $P = 0.024$). * represents a significant value $P < 0.05$; *** represents a significant value $P < 0.001$.

4.1.2 PRDM2 Knock Down Projection Areas

We found a significant effect of PRDM2 KD through BLA projecting neurons in the average freezing along the different tones in the fear test, 24 hours after fear conditioning (Figure 15, $F_{(1,37)} = 4.414$; $P = 0.043$). Specifically, PRDM2 KD rats showed higher freezing than scrambled rats (Figure 15, $F_{(1,37)} = 4.414$; $P = 0.043$). There was a significant effect of the individual tones in the average freezing of each subject (Figure 15, $F_{(1,37)} = 23.187$; $P < 0.001$) suggesting that fear response was different following the first tones.

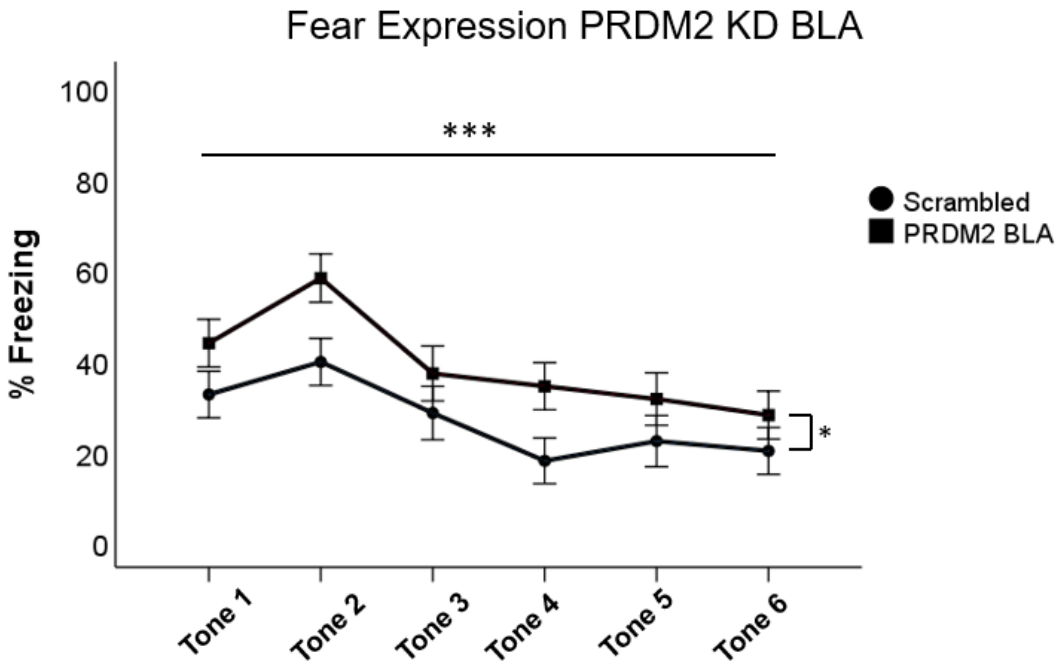

Figure 15: Treatment effect of PRDM2 KD through BLA-projecting neurons in average freezing along repeated tones in fear test 24 hours after conditioning (Repeated-measures ANOVA: within-subjects effect: $F_{(1,37)} = 23.187$; $P < 0.001$; between-subjects effect: $F_{(1,37)} = 4.414$; $P = 0.043$). * represents a significant value $P < 0.05$; *** represents a significant $P < 0.001$.

We did not find any significant effect for PRDM2 KD through PAG-projecting neurons in the average freezing along the individual tones during the fear test 24 hours after fear conditioning (Figure 16, $F_{(1,39)} = 0.409$; $P = 0.526$). Additionally, there was a significant effect of the individual tones in the average freezing within each subject (Figure 16, $F_{(1,39)} = 7.026$; $P = 0.012$) suggesting that fear response was different after the first tones.

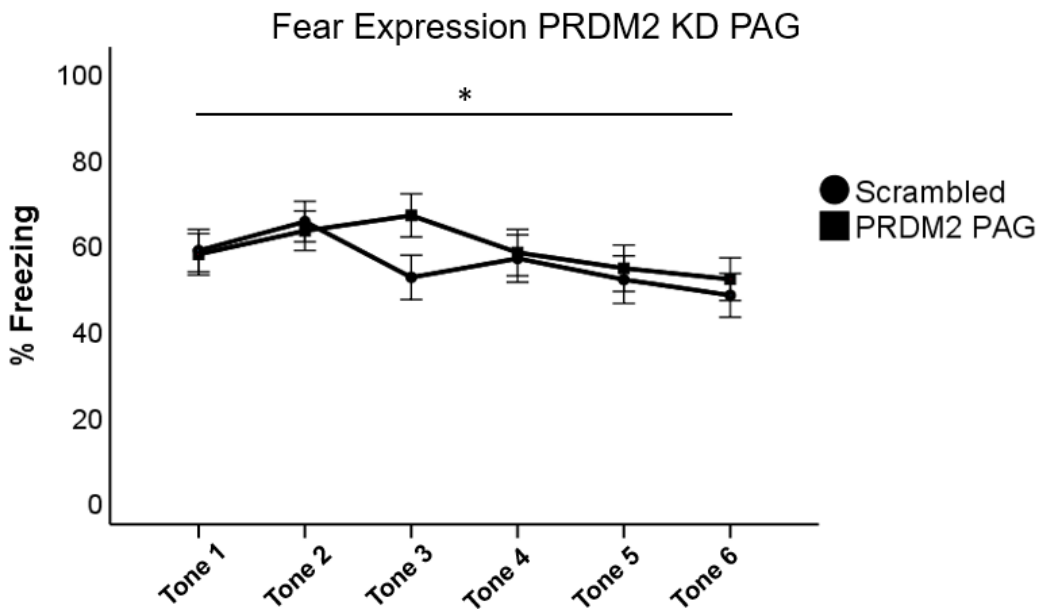

Figure 16: Treatment effect of PRDM2 KD through PAG-projecting neurons in average freezing along repeated tones in fear test 24 hours after conditioning (Repeated-measures ANOVA: within-subjects effect: $F_{(1,39)} = 7.026$; $P = 0.012$; between-subjects effect: $F_{(1,39)} = 0.409$; $P = 0.526$). * represents a significant value $P < 0.05$.

PRDM2 KD through BLA-projecting neurons did not affect either general locomotion (Figure 17a, $F_{(1,33)} = 1.425$; $P = 0.241$) or the relative time spent in the open arms in the EPM (Figure 17b, $F_{(1,37)} = 1.050$; $P = 0.312$). For the latter, we found that variances were not equal by running a Levene's test of homogeneity ($F_{(1,37)} = 8.324$; $P = 0.006$). However, we did not find any significant effect of treatment on the relative time spent in the open arms in the EPM neither with non-parametric Mann-Whitney U test ($U = 176$; $P = 0.708$).

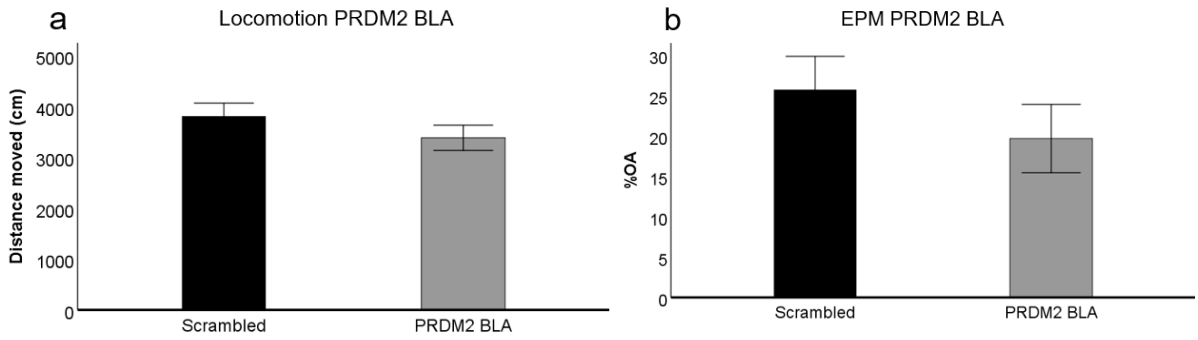

Figure 17: Treatment effect of PL-to-BLA PRDM2 KD over general locomotor activity (a) and relative time spent in the open arms of EPM (b) (One-way ANOVA, locomotion: $F_{(1,33)} = 1.425$; $P = 0.241$; EPM: $F_{(1,37)} = 1.050$; $P = 0.312$).

PRDM2 KD through PAG-projecting neurons did not affect either general locomotion (Figure 18a, $F_{(1,35)} = 0.030$; $P = 0.864$) or the relative time spent in the open arms in the EPM (Figure 18b, $F_{(1,39)} = 0.014$; $P = 0.905$).

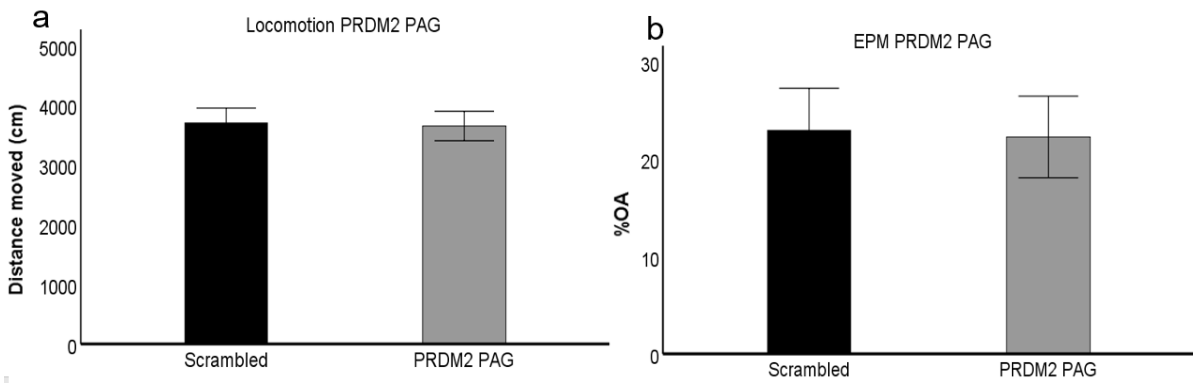

Figure 18: Treatment effect of PL-to-PAG PRDM2 KD over general locomotor activity (a) and relative time spent in the open arms of EPM (b) (One-way ANOVA, locomotion: $F_{(1,35)} = 0.030$; $P = 0.864$; EPM: $F_{(1,39)} = 0.014$; $P = 0.905$).

4.2 Experiment 2

4.2.1 Behavioural assays for anxiety-like behaviour

For the elevated plus maze, we found a tendency for lower relative time spent in the open arms in both stressed and witness groups, respect to control (Figure 19). However, these differences were not significant (Figure 19, $F_{(2,17)} = 0.373$; $P = 0.694$).

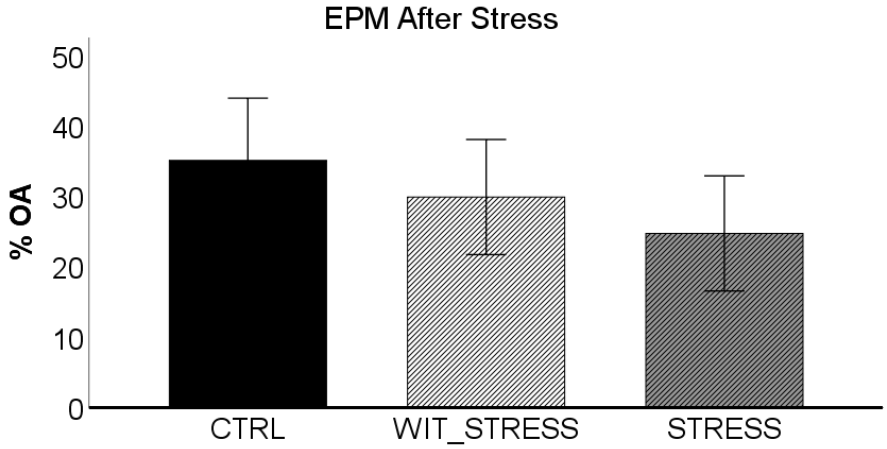

Figure 19: Group effect over relative percentage of time spent in open arms in EPM (One-way ANOVA: $F_{(2,17)} = 0.373$; $P = 0.694$).

We found no difference between the groups in the total locomotor activity in the open field after the stress exposure (Figure 20, $F_{(2,17)} = 0.29$; $P = 0.752$). This was consistent with no differences between the groups at the baseline levels (Figure 20, $F_{(2,17)} = 1.548$; $P = 0.241$).

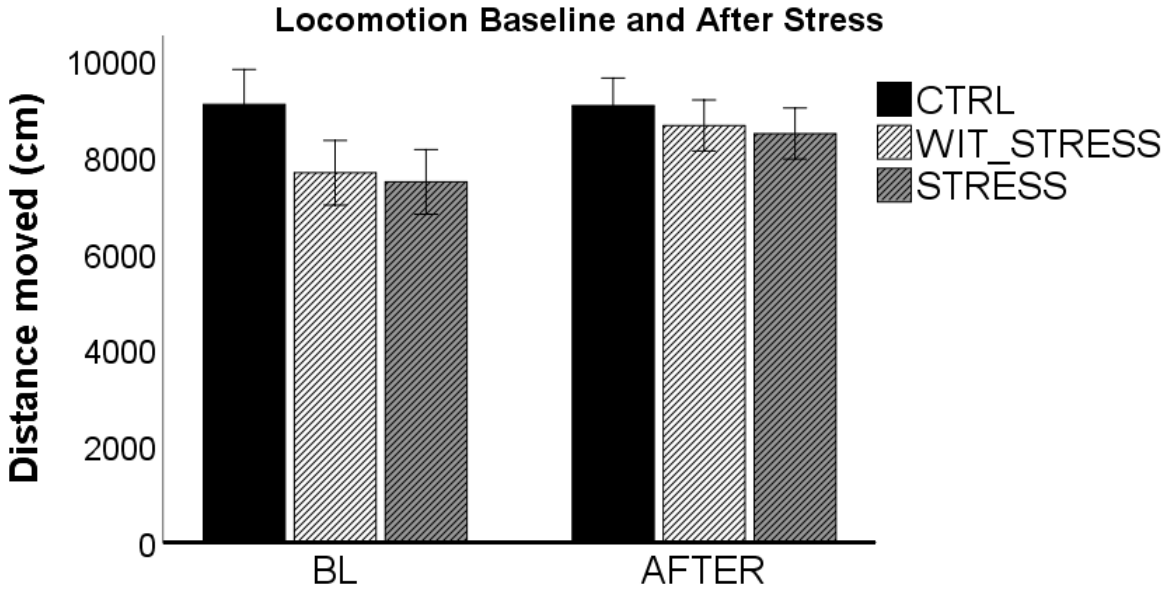

Figure 20: Baseline (BL) and after stress levels for locomotor activity among the groups (One-way ANOVA, BL: $F_{(2,17)} = 1.548$; $P = 0.241$; after: $F_{(2,17)} = 0.29$; $P = 0.752$).

When we analysed the locomotor activity in the open field in five minutes bins, we found a significant effect of the individual bins in the total distance moved, with all the individuals showing higher locomotion in the first five minutes than in the following ones: this was true for both baseline (Figure 21a, $F_{(3,51)} = 91.445$; $P < 0.001$) and after stress time points (Figure 21b, $F_{(3,51)} = 74.841$; $P < 0.001$). However, we found no significant effect of the group neither for baseline (Figure 21a, $F_{(2,17)} = 1.548$; $P = 0.241$) or after stress time points (Figure 21b, $F_{(2,17)} = 0.29$; $P = 0.752$).

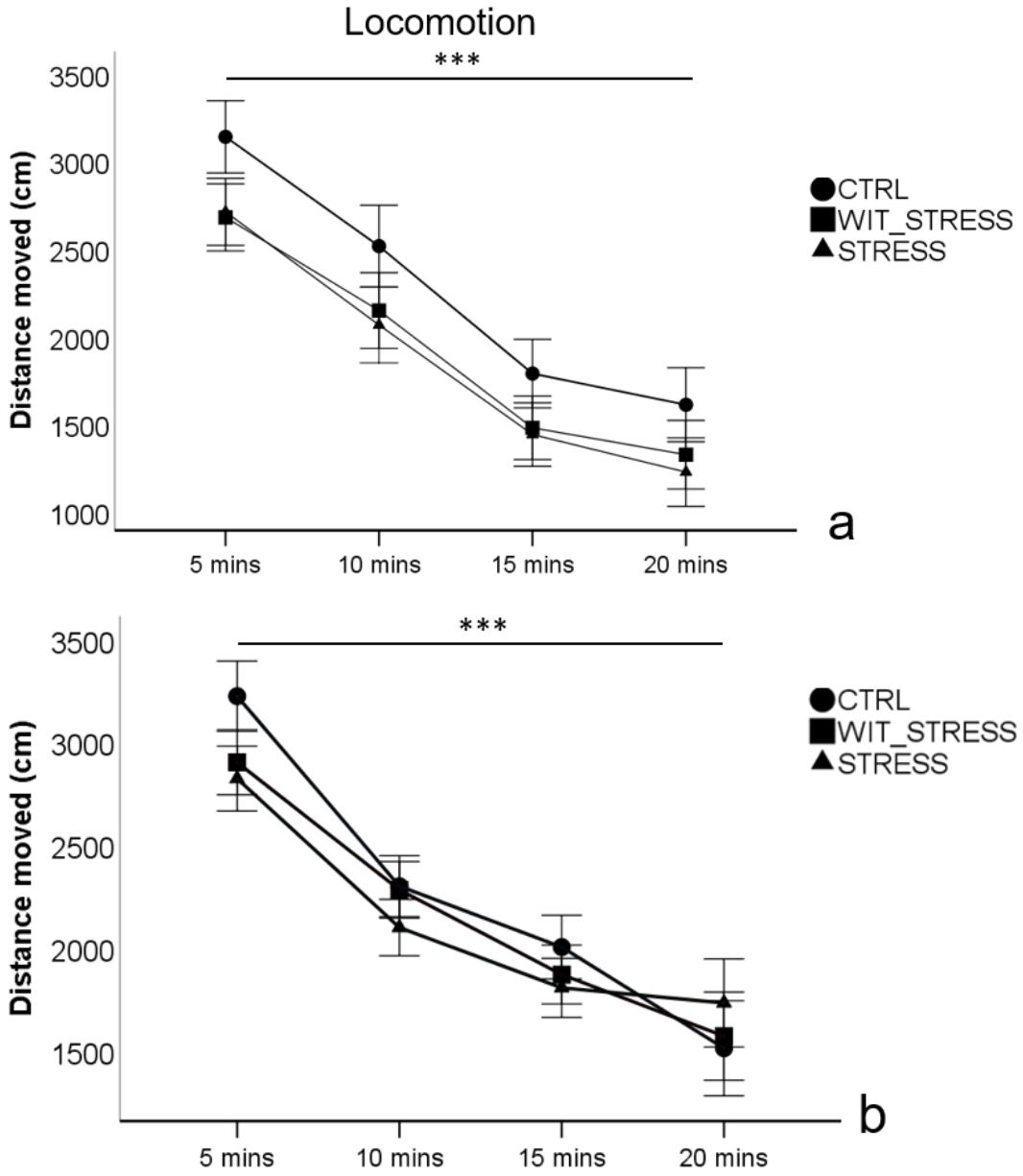

Figure 21: Locomotor activity over five minutes bins for baseline (a) and after stress time points (b) (Repeated-measures ANOVA, within-subjects effect baseline: $F_{(3,51)} = 91.445$; $P < 0.001$; between-subjects effect baseline: $F_{(2,17)} = 1.548$; $P = 0.241$; within-subjects effect after: $F_{(3,51)} = 74.841$; $P < 0.001$; between-subjects effect after: $F_{(2,17)} = 0.29$; $P = 0.752$). *** defines a significant level $P < 0.001$.

In the open field test, we found a tendency for lower percentage time spent in the centre after the stress treatment in both stressed and witness groups, relative to controls (Figure 22). However, there was no significant effect (Figure 22, $F_{(2,17)} = 0.363$; $P = 0.701$). Even at baseline level, we found no significant difference between the groups (Figure 22, $F_{(2,17)} = 1.409$; $P =$

0.271) Compared to baseline, all the groups increased their time spent in the centre after the stress treatment (Figure 22).

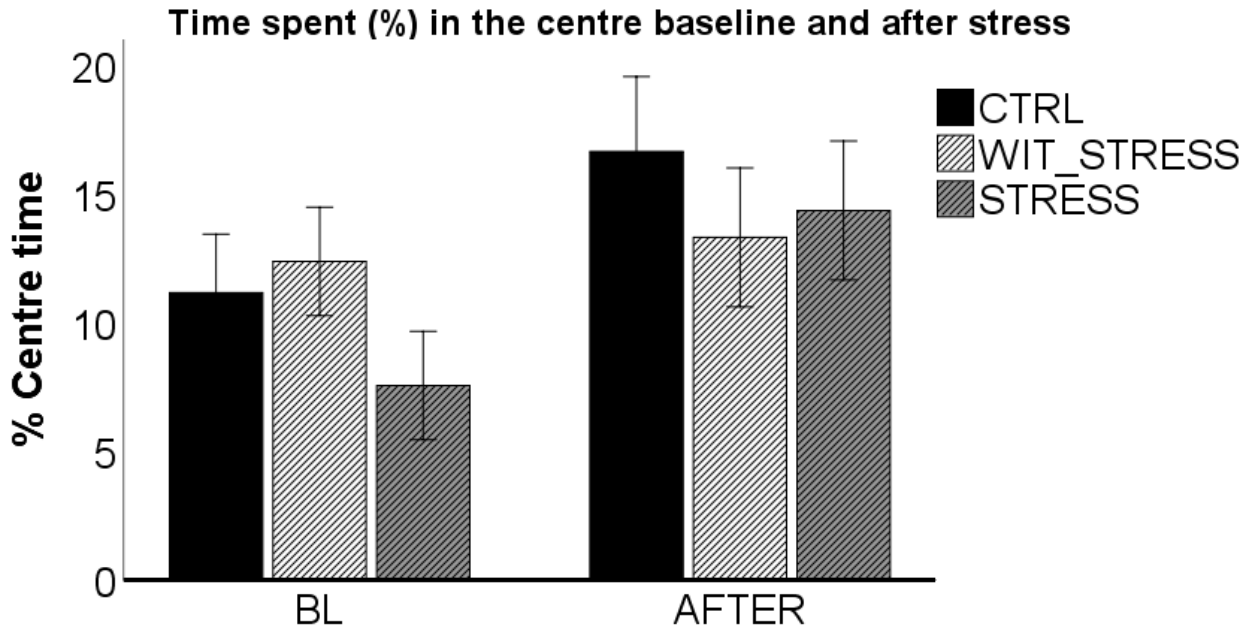

Figure 22: Percentage of time spent in the centre at baseline (BL) and after stress exposure (One-way ANOVA, BL: $F_{(2,17)} = 1.409$; P = 0.271; after: $F_{(2,17)} = 0.363$; P = 0.701).

When we analysed the percentage of time spent in the centre of the open field in five minutes bins, we found no significant effect of the individual bins in the time spent in the centre: this was true for both baseline (Figure 23a, $F_{(3,51)} = 1.845$; P = 0.151) and after stress time points (Figure 23b, $F_{(3,51)} = 2.318$; P = 0.086). Compared to baseline, after the stress treatment, all the groups increased their exploration time in the centre after five minutes from the start of the open field test. However, we saw a tendency of control groups to even increase exploration time in the centre after ten minutes from the start of the open field test, after the stress treatment (Figure 23b). This was not true for stress and witness groups, whose distribution along the bins was almost flat (Figure 23b). However, this tendency was not supported by statistically difference between the groups, neither for baseline (Figure 23a, $F_{(2,17)} = 1.409$; P = 0.271) or after stress time points (Figure 23b, $F_{(2,17)} = 2.172$; P = 0.145).

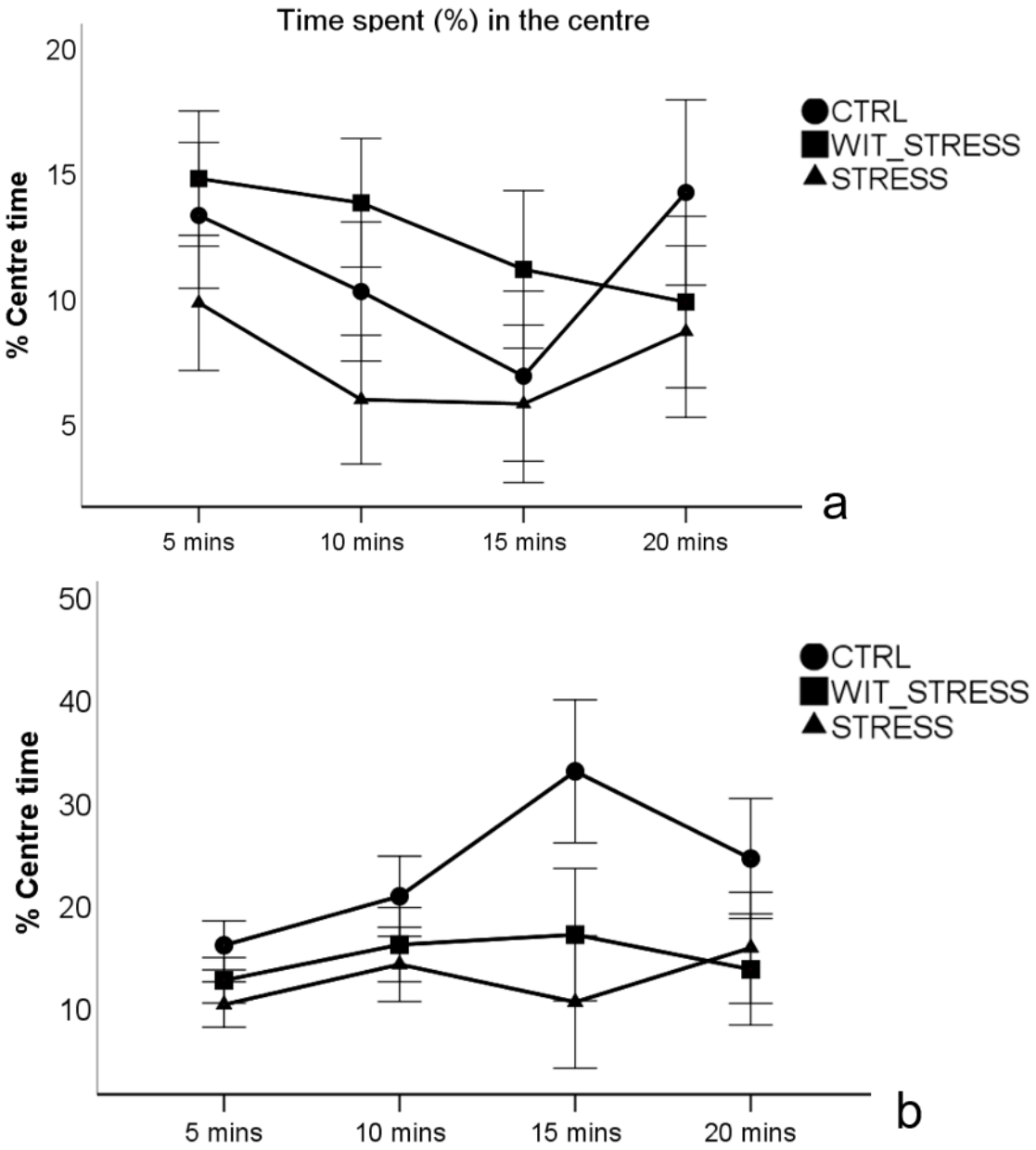

Figure 23: Percentage time spent in the centre along five minutes bins, both at baseline (a) and after stress (b) time points. (Repeated-measures ANOVA, within-subjects effect baseline: $F_{(3,51)} = 1.845$; P = 0.151; between-subjects effect baseline: $F_{(2,17)} = 1.409$; P = 0.271; within-subjects effect after: $F_{(3,51)} = 2.318$; P = 0.086; between-subjects effect after: $F_{(2,17)} = 2.172$; P = 0.145).

4.2.2 Social Interaction Test

In the social interaction test, we found that both stressed and witness groups tended to spend less time in the corners when social target was presented, than controls which spent more time in the corners when social target was presented (Figure 24).This was true after the stress

treatment but not at the baseline (Figure 24). However, we found no significant group effect either at baseline (Figure 24, $F_{(2,17)} = 1.014$, P = 0.384) or after stress treatment (Figure 24, $F_{(2,17)} = 1.019$; P = 0.382).

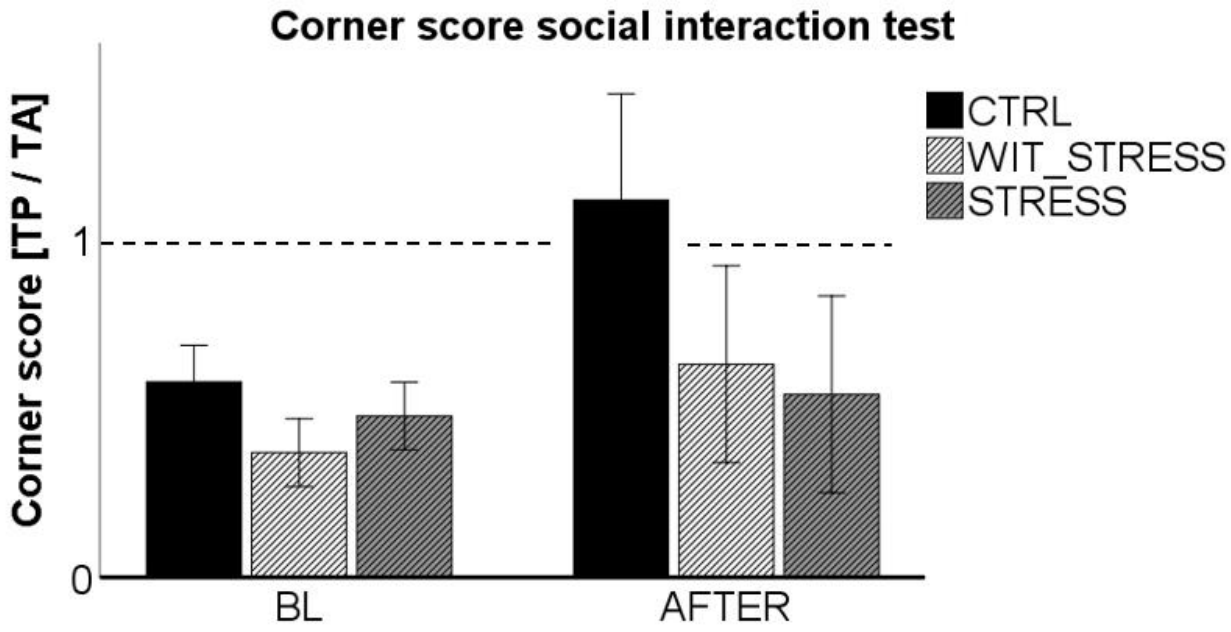

Figure 24: Corner score (Target present (TP) / Target absent (TA)) for baseline (BL) and after stress time points. Dashed line represents time point where TP = TA. All the values above the line represent scores where TP > TA; all the values below the line represent scores where TP < TA (One-way ANOVA, BL: $F_{(2,17)} = 1.014$, P = 0.384; after: $F_{(2,17)} = 1.019$; P = 0.382).

For the interaction score, we found that both stressed and witness groups interacted more with the social target than the controls, after the stress treatment (Figure 25). We did not find similar differences at baseline levels (Figure 25). Indeed, there was a significant effect of the group treatment after stress exposure (Figure 25, $F_{(2,17)} = 5.704$; P = 0.013), but not at baseline levels (Figure 25, $F_{(2,17)} = 0.303$; P = 0.742). As revealed by *post hoc* analyses, we found a significant difference between the stress and the control group (Difference of the means = 0.69; P = 0.027) and between the witness stress and control group (Difference of the means = 0.73; P = 0.019), after the stress treatment. However, there was no significant difference between stress and witness stress groups (Difference of the means = 0.04; P = 0.984).

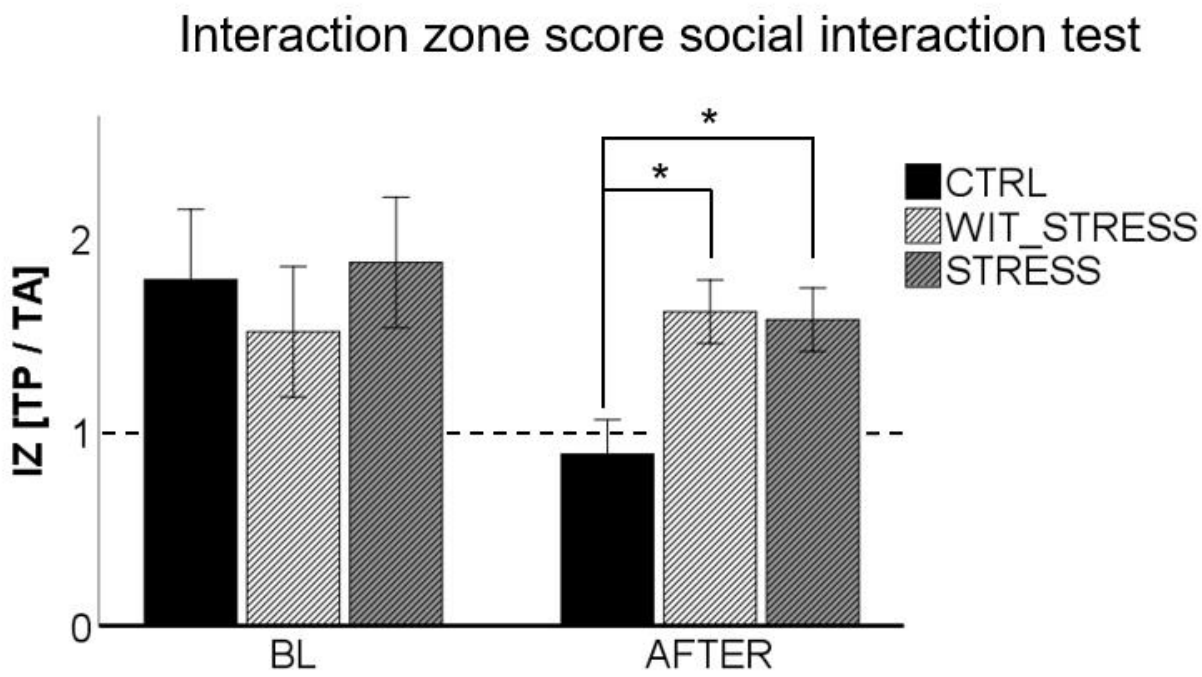

Figure 25: Interaction zone (IZ) scores (Target present (TP) / Target absent (TA)) for baseline (BL) and after stress levels. Dashed line represents time point where TP = TA. All the values above the line represent scores where TP > TA; all the values below the line represent scores where TP < TA (One-way ANOVA, BL: $F_{(2,17)} = 0.303$; P = 0.742; after: $F_{(2,17)} = 5.704$; P = 0.013). * represents a significant level P < 0.05.

4.2.3 Plasma Corticosterone level

For corticosterone level, we found a significant group effect over the plasma concentration after the first session of the foot shock/witness paradigm (S1, Figure 26, $F_{(2,17)} = 5.854$; P = 0.012) but not after the last session of the paradigm (S5, Figure 26, $F_{(2,17)} = 2.221$; P = 0.139) and at baseline (BL, Figure 26, $F_{(2,17)} = 0.188$, P = 0.83). Pairwise comparisons in *post hoc* tests revealed that there was a significant difference in corticosterone between stress and witness stress groups (Difference of the means = 20.075; P = 0.01). However, neither the stress (Difference of means = 14.29; P = 0.086) or the witness group (Difference of the means = 5.78; P = 0.634) differed significantly from the control.

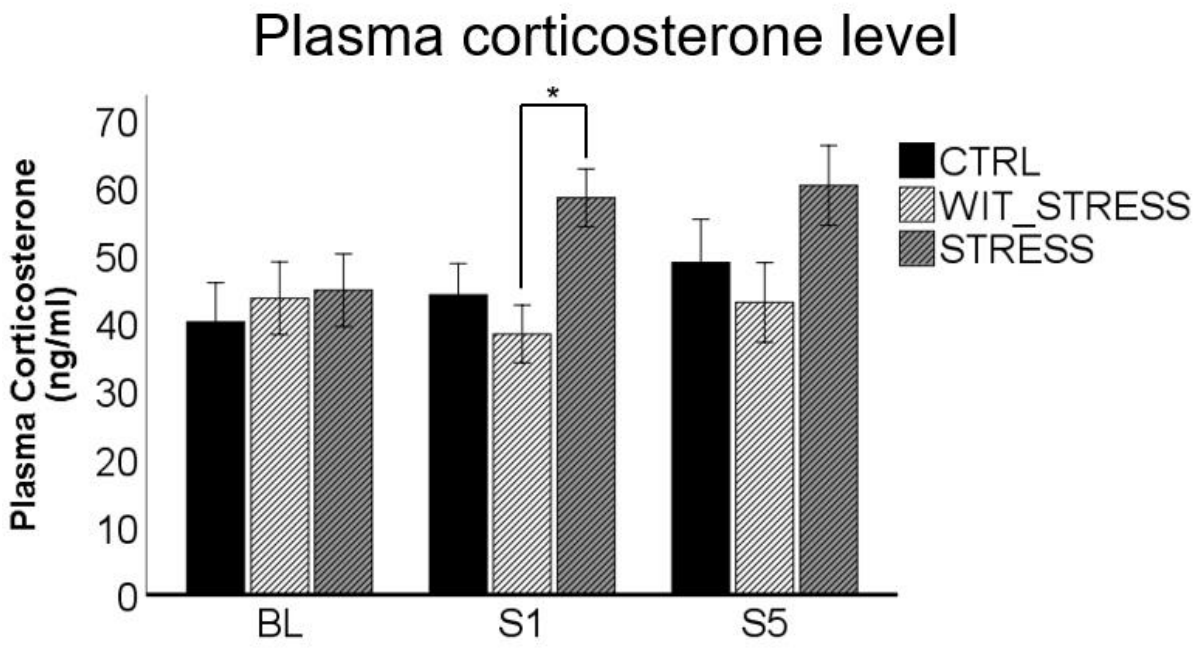

Figure 26: Corticosterone levels along the three time points (baseline (BL), first session (S1) and last session (S5)) (One-way ANOVA, BL: $F_{(2,17)} = 0.188$, P = 0.83; S1: $F_{(2,17)} = 5.854$; P = 0.012; S5: $F_{(2,17)} = 2.221$; P = 0.139). * represents a significant level $P < 0.05$.

5 Discussion

5.1 Experiment 1

The key findings of this study were the followings: (1) PRDM2 Knock Down in prelimbic cortex caused a persistent increased fear expression in the cued fear conditioning paradigm; (2) we found that PRDM2 KD in the PL-BLA projection was sufficient to induce increased fear expression whereas PRDM2 KD in PL-PAG projections did not affect fear expression; (3) none of the treatments affected baseline anxiety-like behaviours either in an open field test or elevated plus maze (EPM).

Downregulating the expression of PRDM2 in the prelimbic cortex of rats lead to increased fear expression in the cued conditioning paradigm. This was consistent with previous works showing the pivotal role of PL in fear behaviours, both in rodent models (Vidal-Gonzalez et al., 2006; Corcoran & Quirck, 2007; Laurent & Westbrook, 2009) and in humans (Milad et al., 2007; Linnman et al., 2011). Inactivation of PL reduced fear expression in rats (Corcoran & Quirck, 2007; Laurent & Westbrook, 2009) whereas microstimulation of PL increased fear

expression (Vidal-Gonzalez et al., 2006). Functional neuroimaging studies showed increased blood oxygen level-dependent (BOLD) signals associated with fear acquisition and expression in the dorsal anterior cingulate cortex (dACC) in humans, an equivalent of rodent prelimbic cortex (Milad et al., 2007). Additionally, we found a similar significant effect of PRDM2 knock down in PL either when fear expression was tested 24 hours after conditioning or one week later. This was consistent with previous studies, showing that PL is crucial in fear retrieval both at early and late time points (Do-Monte et al., 2015). Given these results and given its high expression in prefrontal cortex, we hypothesise that PRDM2 is involved in balancing the top-down regulation of cortical regions over fear-related behaviours, such as freezing, and that its impaired expression can accentuate these behaviours.

Evidence in the field reports that projections from the PL cortex to BLA regulates a variety of fear-related behaviour (Sierra-Mercado et al., 2011; Arruda-Carvalho & Clem, 2014). Above all, PL-to-BLA inputs increases fear expression to conditioned stimuli (Arruda-Carvalho & Clem, 2014; Courtin et al., 2014). BLA is one of the 13 regions that compose the amygdala. BLA is made of three main classes of neurons: principal (excitatory) glutamatergic neurons that compose 80-90 % of BLA; inhibitory GABAergic interneurons that compose 10-20 % of BLA; and neuroglial cells (Pitkanen, 2000). A balance between excitatory and inhibitory outputs mediate the functionality of BLA and its involvement in relevant emotion-related behaviour (Sotres-Bayon & Quirk, 2010). Hyperexcitability of BLA principal neurons increases fear expression, for example enhancing outputs of fear-related behaviour (e.g. freezing) from the central amygdala (Prager et al., 2016). This hyperexcitability can be a consequence of impaired GABAergic inhibitory control over excitatory glutamatergic neurons in BLA (Sharp, 2017). In this respect, our study found an effect of PRDM2 knock down in PL-to-BLA projecting neurons in fear expression in rats. For this reason, we might speculate that downregulating the expression of this enzyme in PL-to-BLA neurons would result in disinhibition of BLA nuclei and consequent enhancement of fear-related behaviour. However, in our study we did not look for specific targets of PRDM2 knock down-effects in the BLA (either glutamatergic or GABAergic neurons) so we do not have enough data to support our speculation.

Another possible mechanism by which PL-to-BLA circuitry might affect fear behaviour is through impaired discrimination of safe stimuli. Intense work from Likhtik and colleagues (2014,2015) showed how mPFC-to-BLA circuitry is involved in the discrimination of safety signals versus aversiveness and so plays an important role in adaptive responses to potential

threats. They suggest that dysregulation of this circuitry will result in exaggerated fear and anxiety-like behaviour in safe situations in rodent models, a condition that parallels humans' symptoms in PTSD and generalized anxiety disorders (Likhtik et al., 2014). In line to this hypothesis, our results suggest that PRDM2 knock down might decrease PL-to-BLA connectivity, resulting in failure of BLA to inhibit fear in safe contexts (e.g. Conditioned Stimuli uncoupled to Unconditioned Stimuli). Nevertheless, this hypothesis might be contrasting the one described above suggesting the hyperexcitability of BLA as a major cause of increased fear expression. However, we cannot exclude that both the mechanisms might cooccur given the heterogeneity of BLA cellular population. For this reason, we need to investigate for possible cellular specificity of PRDM2 effects over fear-related behaviours. Furthermore, we need molecular studies and gene networking approaches to investigate the major targets of PRDM2 epigenetic modifications in these groups of neurons to facilitate the understanding of the mechanisms underlying these complex behaviours.

mPFC-to-BLA connectivity plays a pivotal role in innate anxiety too (Likhtik et al., 2014;2015). However, this was not supported by our study where PRDM2 knock down in PL-to-BLA neurons did not affect baseline anxiety either in the open field or the elevated plus maze. We did not find any significant effect of PRDM2 knock down through PAG-projecting neurons. This was in contrast with evidence for an important role of periaqueductal grey in mediating fear behaviours (LeDoux et al., 1988; Vianna et al., 2001). However, a possible explanation for our results resides on the fact that other neuronal pathways recruiting PAG are involved in fear-related behaviours, and not specifically PL projecting neurons (Penzo et al., 2014). Central amygdala activation may act to disinhibit PAG neurons, driving increased fear expression in rats (Penzo et al., 2014) and in mice (Tovote et al., 2016). Instead, there is little evidence in the literature about direct effects of PL neurons projecting to PAG in fear-related behaviours, even if direct projections of PL neurons to PAG are well documented (Vianna & Brandao, 2003). Supporting this, principal neurons from the PL evoked by presentations of auditory conditional stimuli associated with foot shocks, preferentially targeted BLA rather than PAG to drive fear expression in mice (Courtin et al., 2014). Another possible explanation resides on the fact that PAG involvement in fear behaviours is well documented in contextual fear conditioning paradigm (Leman et al., 2003; Borelli et al., 2013) but not in cued conditioning paradigm. There is intense work reporting how these two paradigms, even if based on the same CS-US associations, recruit different neuronal pathways (Goosens & Maren, 2001).

Contextualizing our results to the pathophysiology of a comorbid phenotype of anxiety-like disorders and alcohol use disorders, PRDM2 KD effects over fear expression are in alignments with previous findings. Indeed, PRDM2 KD in dmPFC caused a typical phenotype for alcohol dependence in rats, such as impaired control over alcohol drinking, compulsive-like drinking and withdrawal-induced anxiety-like behaviour (Barbier et al., 2016). Moreover, given the evidence for the role of mPFC-to-BLA circuitry in alcohol use disorders (Tye et al., 2013; Rau et al., 2015), our results support the evidence for neuronal overlaps between AUDs and anxiety-like disorders, even at a molecular level. Specifically, a recent study showed that chronic intermittent ethanol (CIE) exposure caused an increased glutamatergic activity from PL-to-BLA that lead also to increased anxiety-like behaviour associated with withdrawal (McGinnis et al., 2019). Unfortunately, in our studies we did not target specifically glutamatergic neurons of the PL, but we cannot rule out the possibility that PRDM2 knock down mediates its effect in alcohol drinking- and fear-related behaviour by dysregulating this specific circuitry.

Despite the contradictory behavioural outcomes of alcohol addiction (rapid movement and goal-directed behaviour) and fear response (inhibition of movement, e.g. freezing), similar associative responses and psychological processes can be recruited, justifying the neuronal overlaps between AUDs and anxiety-like disorders (Giustino & Maren, 2015). Indeed, studies showed how rats that were highly responsive to food predictive cues, also showed increased auditory fear, suggesting that they were 'cue-directed' (Morrow et al., 2015). Moreover, in support to this, different neuronal populations in the PL can convey different behaviours even if recruiting similar afferent and efferent connections (Halladay & Blair, 2015).

It is important to clarify that the results presented in this report refer exclusively to male individuals. Indeed, we cannot rule out the possibility of sex-specific mechanisms by which prelimbic cortex regulates fear behaviour. For instance, despite the consistent dichotomy between PL and IL functions in fear expression and fear extinction respectively, studies reported that activation of PL is fundamental for fear extinction in female rats (Fenton et al., 2014). Dimorphic plasticity of mPFC consolidates the importance for direct studies investigating the neurological basis for differential susceptibility to anxiety disorders (Maeng et al., 2010; Farrell et al., 2013). Moreover, we did not include clustering analysis to our study to investigate for possible interindividual differences within the treatment groups. Retrospective behavioural analyses of subpopulations of high freezing vs low freezing rats showed different structural IL-to-BLA projections in male rats (Gruene et al., 2015). Understanding the

neurological basis for interindividual differences and either if they are pre-existing or modulated by the learning and fear experiences, remains one of the major challenges in the field.

In conclusion, we showed evidence for a role of a histone methyltransferase, PRDM2, in regulating fear behaviours through top-down control of PL cortex over afferent neuronal areas. Specifically, BLA-projecting neurons were mostly involved in these effects, rather than PAG-projecting neurons. This parallels findings that PRDM2 dysregulation is associated to alcohol use disorders, providing a possible target for comorbid phenotypes with anxiety-like disorders. In order to elucidate the mechanisms behind this phenotype we need to study the molecular targets of PRDM2 epigenetic modifications and eventual cell-specific effects in the subpopulations of PL cortex and BLA. Sex-specific effects and interindividual differences need to be investigated.

5.2 Experiment 2

The key findings of our study were the followings: (1) There was no significant effect of the stress treatment over anxiety-like behaviour, either in the EPM or in the open field test; (2) stressed and witness groups spent more time interacting with a social stimulus in a social interaction test than control groups after a stressful experience, with no difference between stressed and witness groups; (3) the stressed group showed a higher corticosterone level than witnesses but only after the first stress exposure.

We found that the foot shock/witness paradigm did not affect anxiety-like behaviour in validated behavioural assays, such as EPM and open field test, even if the stressed groups showed a tendency to higher anxiety-like behaviour than controls. This was in contrast with previous studies that reported that exposure to repeated inescapable foot shocks and witnessing conspecifics exposed to foot shock lead to anxiety-like behaviour in rodents (Van der Berg et al., 1998; Pijlman et al., 2002,2003; Bali & Jaggi, 2015; Verma et al., 2016). We found that both physically stressed rats and witnesses showed a similar tendency for anxiety-like behaviour in respect to controls. This was in contrast with previous findings reporting differential behavioural responses following physical or psychological stressor (Pijlman et al., 2003; Mousavi et al., 2019). Indeed, evidence reports that physically stressed rats are hypoactive in an open field test, whereas witnesses tend to be hyperactive in an open field test

(Pijlman et al., 2003; Mousavi et al., 2019). Differential behavioural responses are also coupled to distinct cardiovascular and inflammatory consequences to the two types of stressors (Finnel et al., 2017), reflecting the fact that different neuronal circuitry is involved in processing stress-induced effects (Kavushansky et al., 2009). However, we were not able to replicate these findings in our study. There are several possible explanations for this: first of all, the presence of a former cage mate witnessing the stressful experiences might have played a social buffering effect, given the evidence of social empathy in rats (Church, 1959); specifically, social support is even more effective in reducing stress-induced effects if the partner is näive to any prior stressor (Kiyokawa et al., 2004, Atsak et al., 2011). Secondly, the intensity and the duration of the foot shock were not sufficiently high to replicate previous findings (Bali & Jaggi, 2015). Lastly, the number of individuals per group was quite low and this caused underpower for statistically evidence. To implement our model, we need additional studies to assess the right conditions of the stressor to induce long-term anxiety-like behaviour both in stressed subjects and witnesses and to better investigate for possible differential effects.

Moreover, in the social interaction test we found that both foot shock-stressed and witness rats showed higher interaction score and a tendency to lower corner scores than controls after the stressor exposure. In this case witnesses showed similar results to stressed subjects. These results suggest that there was an increased interest towards unknown social stimuli after a stressful experience, in stressed and witness groups relative to controls. This was contrasting from previous work that showed social avoidance after both social stress (e.g. social defeat stress) and electric foot shocks (Haller & Bakos, 2002; Leveleki et al., 2006; Lukas et al., 2011). Warren et al. (2013) reported that witnessing the defeat of a conspecific induced long lasting social avoidance behaviour in mice. However, in our lab we found increased social interaction in social defeated rats (Not published), similarly to what we found in the foot shock/witness paradigm. However, there are no replicates in the literature reporting the effect of witnessing foot shock stress in social behaviour. We hypothesise that our results might reflect an increased motivation in stressed subjects towards social stimuli. This is even supported by studies showing that socially isolated rats have increased motivation to interact with social stimuli (Hol & Spruijt, 1992, Hol et al., 1999). However, this result could have been confounded by a decreased motivation of controls to interact with unknown social stimuli. Indeed, they were continuously interacting with their former cage mates for one hour per day. Studies reported lower motivation to social stimuli in group-housed rats than individually housed ones (Van der

Berg et al., 1999). We need to control this condition in future studies, maybe with more stringent routines for social interaction between former cage mates in control groups.

Lastly, we found a significant rise in plasma corticosterone level in the stressed group relative to the witnesses but not to controls and only after the first stress exposure. However, this pattern was consistent even after the last stress exposure, even if the corticosterone levels did not differ significantly among the groups. Indeed, we report that physically stressed but not witnesses showed higher corticosterone levels relative to controls after the first and the last exposure to the stressor. This might suggest that the witnessing stress failed to induce relevant effects in plasma corticosterone levels, differently to what was reported in similar studies in the past (Chrousos & Gold, 1992; Mousavi et al., 2019). This might question the effectiveness of this type of stressor in our model; however, given that corticosterone is a metabolic hormone sensitive to physical activity (Hill et al., 2008), we cannot rule out the possibility that the differences among stressed and witness groups might reflect a differential physical activity during the stress exposure rather than ineffective stress induction. Indeed, the box where the witnesses were forced to observe their former cage mates exposed to inescapable foot shocks was much smaller than the box where both stressed and control groups have been put during the test. The different sizes of the boxes unavoidably lead to different possibilities for the rats to actively move around and explore the new environment during the test; this might have contributed to lower corticosterone levels in witnesses than controls and stressed rats. However, we do not have enough data to support this speculation. Indeed, we need to better control for the possible confounding of the sizes of the boxes in future studies, to disentangle the actual effects of witnessing stress to the effects of limited movement over both behavioural and physiological endpoints. A possible solution is to include an additional experimental group of animals that will be introduced to the small boxes for the same amount of time, but without witnessing any traumatic event.

In conclusion, our foot shock/witness paradigm was not able to replicate the differential effects of physical and psychological stress in the open field and elevated plus maze tests, showed in previous studies (Pijlman et al., 2002,2003). We showed for the first time a stress-induced increased social interaction towards a novel conspecific in rats, even if we cannot rule out a possible confounding from our experimental set up. Future studies need to focus on more accurate experimental designs to implement a model that has the potentiality for studying psychosocial stress and its involvement in alcohol dependence. Moreover, despite its

questionable ethological relevance, this model might represent a valid alternative to other social stressors (e.g. social defeat stress), given its controllability over stressor intensity, and above all its sensitivity to sex-specific effects (Viviani et al., 2012; Mousavi et al., 2019).

6. Societal and ethical considerations

Alcohol use disorders represent one of the major risks for health problems, accounting for 5.9 % of overall deaths in 2012 (WHO, 2012). Despite the society tends to consider other recreational drugs (e.g. nicotine, heroin, cocaine) as major burdens for the society, alcohol use disorders are more prevalent than any other substance abuse (Falk et al., 2008; WHO, 2012). The societal impact of this situation is relevant, not only considering the health care economic costs (Parker et al., 1987) but also related to crime-related costs (Cartwright, 1999, Lynch et al., 2010). The impact of alcohol abuse in domestic violence and other social problems is reported (Rossow, 2000, Perugi et al., 2002). Moreover, psychiatric disorders, such as social phobia and major depression are comorbid with alcohol use disorders (Perugi et al., 2002, Sullivan et al., 2005).

In this respect, biomedical research is focusing its interest over prevention strategies and possible treatments for AUDs. However, some of the clinical aspects of alcohol abuse cannot be replicated in human studies due to technical and ethical issues (Spanagel, 2003). So, researchers have employed animal models, especially rodents, to overcome this problematic (Spanagel, 2003). Indeed, rodent models allow controllable variables in experimental set-ups, where the behaviour of the subjects can be manipulated (Lynch et al., 2010). However, modelling the complex phenotype of alcohol addiction in animals is challenging (Stephens et al., 2011). Many researchers in the field tried to find consilience of human and rodent phenotypes to parallel laboratory experiments to the clinics (Crabbe et al., 2010, Heilig et al., 2010). For instance, even if animals consume ethanol naturally as a component of fruits (Dudley, 2002), they rarely self-administer alcohol levels that mimic behavioural intoxication (Dole et al., 1985, Crabbe et al., 2010). Although rodents can be easily trained to ‘accept’ higher ethanol concentration, despite its aversive taste (Grant & Samson, 1985), this will still question the translational value of rodents’ phenotype to the human condition. Indeed, the viability of these models requires that the pathological condition in humans parallel a natural, and not artificial, pathological condition in animal models (Solomon, 2017). In addition to the translational issues, these models inevitably cause ethical issues. Addiction research is indeed

one of the main targets of animal rights activities (Lynch et al., 2010). Drinking high ethanol concentrations and experiencing non-natural stressors (e.g. electric foot shocks) do not belong to the natural behavioural repertoire of these species (Stephens et al., 2011). This is an issue, given the recent increased interest for animal welfare, especially for laboratory animals (Baumans, 2005).

For this reason, we need to focus on more ethologically relevant methods to increase the predictive value of animal models for the human situation, trying at the same time to minimise and reduce animal suffering in experimental procedures.

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Appendix (Experiment 1)

Fear Study 1

Treatment	Tone1	Tone2	Tone3	Tone4	Tone5	Tone6	Average Tone 1 + 2
Scrambled	41.3±6.7	40 ± 5.2	37.9±6.6	42.8±5.5	40.9±6.1	42.2±5.8	40.6±5.4
PRDM2	57.5±4.8	59.2±5.5	50.8±5.4	55.7±5.8	57.2±5.7	49.5±5.7	58.3±4.07

Anxiety-like behaviour Study 1

Treatment	Locomotor activity	% time spent open arms
Scrambled	4682,736 ± 1338.95	40.6 ± 5.1
PRDM2	5076.535 ± 1647.437	31.6 ± 5.7

Fear Study 2

Treatment	Tone1	Tone2	Tone3	Tone4	Tone5	Tone6	Average Tone 1 + 2
Scrambled	50±5.9	38.9±8.4	30± 7.6	26.7±7.7	25.6±6.4	24.2±8.3	44.4±6.6
PRDM2	66.7±5.7	60.8±8.6	60 ± 7.6	51.4±8.8	32.2±7.3	38.6±6.3	63.7±5.4

Fear study 3

Treatment	Tone1	Tone2	Tone3	Tone4	Tone5	Tone6	Average Tone 1 + 2
Scrambled BLA	32.4±5.3	39.6±5.7	28.3±5.5	17.8±4.5	22.2±5.2	20 ± 5.5	36 ± 5.1
PRDM2 BLA	43.7±4.9	58 ± 4.5	37 ± 6.3	34.2±5.6	31.4 ± 6	27.9±4.8	50.8 ± 4.5
Scrambled PAG	58.2 ± 5	64.9±4.9	51.9±5.8	56.3±5.8	51.4±6.1	47.8 ± 5	61.5 ± 4.5
PRDM2 PAG	57.3±4.7	62.8±4.4	66.3±4.3	57.7±5.1	54 ± 4.8	51.5 ± 5	60 ±4.1

Anxiety like behaviour Study 3

Treatment	Locomotor activity	% time spent open arms
Scrambled BLA	3819.2 ± 292.9	25.8 ± 5
PRDM2 BLA	3392.2 ± 210.	19.7 ± 2.9
Scrambled PAG	3706.6 ± 239.4	22.9 ± 3.8
PRDM2 PAG	3646.8 ± 248.9	22.2 ± 4.6

Appendix (Experiment 2)

EPM

Treatment	% time spent open arms
Control	35.2 ± 10.7
Witness	29.9 ± 7
Stress	24.8 ± 7.6

Open Field Locomotor activity Baseline

Treatment	5 mins	10 mins	15 mins	20 mins	Total
Control	3149.1±100.3	2526.1±281.2	1799.5±206.5	1621.7±206	9096.3±738.2
Witness	2689.7±178.4	2158.8±189.1	1490.3±211.7	1337.2±272.8	7676±705.7
Stress	2721.3±283.5	2076.5±228.5	1452.1±166.6	1237.8±127.6	7487.7±732.5

Open Field Locomotor activity After Stress

Treatment	5 mins	10 mins	15 mins	20 mins	Total
Control	3232.6±109.9	2307.8±110.4	2011.4±121.3	1519.1±85.6	9070.9±366.2
Witness	2910.9±178.1	2290.1±159.4	1877.8±184.7	1578±271.4	8656.7±593.3
Stress	2831.3±201.5	2106.7±163.5	1813±147.2	1739.4±268.4	8490.5±679.1

Open Field Centre time (%) Baseline

Treatment	5 mins	10 mins	15 mins	20 mins	Total
Control	13.3 ± 2.6	10.3 ± 3.3	6.9 ± 2.5	14.2 ± 2.9	11.2 ± 2.3
Witness	14.8 ± 3.5	13.8 ± 2.9	11.2 ± 5	9.9 ± 3.7	12.4 ± 2.5
Stress	9.8 ± 2.5	6 ± 2.1	5.8 ± 1.7	8.7 ± 4.2	7.6 ± 2

Open Field Centre time (%) After Stress

Treatment	5 mins	10 mins	15 mins	20 mins	Total
Control	12.7 ± 1.8	15.7 ± 0.9	24.5 ± 8.8	13.8 ± 3.2	16.7 ± 3.1
Witness	10 ± 0.9	15.3 ± 1.9	14.4 ± 4.1	13.7 ± 3.9	13.3 ± 1.6
Stress	14 ± 3.2	13.7 ± 3.8	11.5 ± 3.8	18.3 ± 5.8	14.4 ± 3.4

Social Interaction Test Baseline

Treatment	Corner Score	Interaction Score
Control	0.6 ± 0.1	1.8 ± 0.3
Witness	0.4 ± 0.1	1.8 ± 0.1
Stress	0.5 ± 0.1	1.9 ± 0.5

Social Interaction Test After Stress

Treatment	Corner Score	Interaction Score
Control	1.1 ± 0.5	0.9 ± 0.1
Witness	0.6 ± 0.2	1.6 ± 0.3
Stress	0.5 ± 0.1	1.6 ± 0.1

Corticosterone level

Treatment	CORT Baseline	CORT Session 1	CORT Session 2
Control	40.2 ± 4.7	44.2 ± 3.9	49 ± 4.2
Witness	43.7 ± 5.7	38.4 ± 2.4	43.1 ± 4.4
Stress	44.9 ± 5.7	58.5 ± 5.9	60.4 ± 8.1