Early life stress induces persistent alteration in endocannabinoid system and leads to dysfunctional modulation of emotional memory retrieval

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All my gratitude for Professor Benno Roozendaal...

...whose definition of success inspires the courage to seek beyond it.

Abstract

Early life stress (ELS) is one of the best characterised risk factors for later development of stress-related disorders, such as post-traumatic stress disorder (PSTD). A hallmark feature in PTSD is persistent, uninhibited retrieval of emotional memory. Recent evidence from this lab indicated that glucocorticoids interact with the endocannabinoid system, particularly 2-arachidonoyglycerol (2-AG), to impair the retrieval of emotional memory under stress. Given that adult rats with ELS history show an inability to upregulate 2-AG signalling in hippocampus after acute stress, we hypothesized that glucocorticoids will not impair emotional memory retrieval in ELS animals, whereas a direct augmentation of hippocampal 2-AG signalling will. We first showed that the well-established limited nesting paradigm resulted in fragmented maternal care and elevated plasma corticosterone levels in pups. At adulthood, we trained male offspring on a contextual fear memory paradigm. One hour before retention testing, 24 hours after training, rats were injected with corticosterone (CORT, 3 mg/kg) systemically or administered the 2-AG hydrolysis (MAGL) inhibitor KML-29 (0.2 µg/0.5 µL) directly into the hippocampus. Unlike control rats, we found that systemic CORT injection did not impair retrieval of contextual fear memory in ELS animals. By contrast, direct hippocampal administration of KML-29 impaired memory retrieval in both ELS and control rats in a CB1 receptor-dependent fashion. Thus, these findings support our hypothesis that the inability of ELS rats to modulate memory retrieval under stress might originate from their inability to mount a 2-AG response. Our findings are highly relevant for informing future studies on the link between ELS and maladaptive stress coping and the increased risk for stress-related psychopathologies.

Keywords

Early life stress, endocannabinoid system, 2-AG, glucocorticoids, contextual fear memory retrieval, limited-nesting paradigm

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Chapter 1 Introduction

Early postnatal days of life are a critical period of development, characterized by numerous transformations in the rapidly growing brain (Stiles & Jernigan, 2010). These processes include alterations in neuroendocrine systems essential for the regulation of stress, such as glucocorticoids, corticotrophin-releasing factor, hypothalamus-pituitary-adrenal (HPA) axis function, hippocampal plasticity, and related cognitive functions (McEwen, 2008; Maniam, Antoniadis, & Morris, 2014; van der Kooij, Grosse, Zanoletti, Papilloud, & Sandi, 2015). Thus, the developing brain is especially vulnerable to the detrimental effects of stress in early life (Bale et al., 2010; van der Kooij et al., 2015; Lupien et al., 2009).

Unfortunately, up to one third of individuals worldwide suffer from traumatic, and sometimes abusive, parental care in early life (Labonté et al., 2012). Traumatic care in early life can alter behavioural and physiological responsiveness to the environment and may ultimately lead to the development of a life-long vulnerability to stress-related disorders (Binder et al., 2008; Mehta & Binder, 2012; McEwen, 2003). Perhaps, of all the stress-related disorders reported in clinics, one of the most consequential is post-traumatic stress disorder (PTSD) (Yehuda et al., 2015). A history of trauma in early life is reported as the most consistent risk factor for later development of PTSD (Delahanty & Nugent, 2006; Stevanović, Frančišković, & Vermetten, 2016; R Yehuda, Halligan, & Grossman, 2001). Symptoms related to dysregulations of emotional memory modulation lie at the core of PTSD symptomatology. In fact, two of the four diagnostic criteria of PTSD are related to an inability to suppress intrusive over-recall of traumatic memories; these include re-experiencing of traumatic events as spontaneous flashbacks or recurrent dreams, and an avoidance of external reminders of the events (American Psychiatric Association, 2013). The other two criteria, negative affect and hyperarousal, may well be an effect of the dysregulated memory processing seen in PTSD.

For years, patients disillusioned by the lack of effective treatment options against PTSD symptoms have turned to the consumption of cannabis for symptom relief (Cornelius et al., 2010; Elliott, Golub, Bennett, & Guarino, 2015; VMCA, 2010). The active constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), exerts its effect on the central nervous system through an action on cannabinoid receptors present in brain. These receptors, termed the cannabinoid receptor type 1 (CB1) and 2 (CB2), are part of the intricate neuro-modulatory system innate to stress homeostasis (M. N. Hill et al., 2010). Unlike other neurotransmitters, the endogenous cannabinoid (endocannabinoid) ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are synthesized on demand postsynaptically and released into the synaptic terminal to act in a retrograde fashion on the CB receptors. AEA and 2-AG are hydrolysed intracellularly by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAG-L), respectively (Hillard, 2000; Wilson & Nicoll, 2002). Activation of the CB1 receptor causes a suppression in neurotransmitter release, predominantly GABA, through a $G_{i/o}$ dependent mechanism (Freund, Katona, & Piomelli, 2003; Younts & Castillo, 2014).

Recent evidence implicates the endocannabinoid system in pathophysiology of stress-related disorders, including PTSD. Studies report that alterations in endocannabinoid levels, particularly in conjunction with alterations in plasma cortisol levels, accurately predict 90% of the PTSD cases (Fraser, 2009; Hauer et al., 2014; McLaughlin et al., 2013; Neumeister, 2013). In addition, a reduction in the endocannabinoid levels were found to be associated with the severity of the PTSD symptoms (Wilker et al., 2016). Furthermore, the distribution of CB1 receptors in the brain is found to be altered in individuals with PTSD (McLaughlin et al., 2013; Neumeister, 2013). Moreover, administering patients suffering from PTSD with a synthetic cannabinoid has been reported to decrease daytime flashbacks and nightmares (Fraser, 2009). Thus, the endocannabinoid system may have a role in modulation of memory systems and related neurocognitive functions reported to be aberrant in PTSD.

Acute stress has long been known to impair the retrieval of hippocampal-dependent memories under allostatic conditions (D. J. de Quervain, Roozendaal, & McGaugh, 1998), and this effect of stress is mediated via glucocorticoids (P. Atsak, Roozendaal, & Campolongo, 2012). A substantial body of research has lately identified a close link between the glucocorticoid system and the fast-acting endocannabinoid system (P. Atsak, Roozendaal, & Campolongo, 2012; M. N. Hill et al., 2010; M. N. Hill & Tasker, 2012). There is a substantive

evidence to suggest that the fast, non-genomic effects of glucocorticoids on emotional memory modulation are mediated via an interaction with the endocannabinoid system (P. Atsak et al., 2012; P. Atsak et al., 2015; Campolongo et al., 2009). Moreover, the endocannabinoid system is also implicated in the early life stress (ELS)-induced alterations to the normal development of the HPA axis and is itself affected as a consequence of trauma during early life (Lee & Gorzalka, 2012; Marco et al., 2014).

Collectively, these findings draw a possible link between ELS, vulnerability to adult stress-induced dysfunctions, and a maladaptive stress memory regulation to the endocannabinoid system. Thus, it is crucial to investigate the effects of trauma in early life on persistent alterations in the endocannabinoid system. This may help to identify a possible mechanism through which trauma and stress during early life leads to altered regulation of stress response system in adulthood and neurocognitive dysfunctions, particularly those connected to maladaptive emotional memory regulation.

Previously, Atsak et al. showed that a systemic injection of corticosterone (CORT), administered prior to retention testing, increases hippocampal concentrations of 2-AG, but not AEA, within the same time frame as the retention test. Moreover, direct activation of CB receptors with its agonist WIN55,212-2 in the hippocampus prior to retention testing, impaired retrieval of contextual fear memory. On the other hand, blocking the CB1 receptor while concomitantly administering CORT, prevented the CORT effect on impairment of fear memory retrieval (P. Atsak, Hauer, et al., 2012). These findings suggest that acute stress induces a 2-AG response in the hippocampus, and that CORT interacts with hippocampal 2-AG to impair memory retrieval via a CB1 receptor-dependent mechanism. Most importantly, unpublished findings from Atsak et al. show that adult rats with a history of ELS do not have an adequate 2-AG response to acute stress.

Based on these findings, we hypothesised that in adult rats with a history of ELS, CORT will not be able to impair contextual memory retrieval. Whereas, a direct augmentation of the 2-AG response in itself will be sufficient to impair the retrieval of contextual memory in both ELS animals and controls. Here, we employed, and validated, a limited nesting (LN) model in order to induce chronic ELS in rats. Further, we pharmacologically manipulated the glucocorticoid and endocannabinoid system in order to test our hypothesis in adult rats with ELS using a contextual fear memory (CFC) paradigm.

Chapter 2 Methodology

2.1 SUBJECTS

A total of 84 dams and 42 male Sprague-Dawley rats of three months of age were acquired from Charles River Breeding Laboratories (Kisslegg, Germany). Rats were allowed to habituate for 10 days to the animal facility prior to mating. For breeding, each male was housed together with 2 females for 10 days in standard plastic rat cages (43x27x16 cm) and maintained under standard housing conditions (22°C, 55% humidity, *ad libitum* access to food and water), in a 12/12-hour light/dark cycle (lights on 07:30 until 19:30). Males were then removed from the cages to allow dams to habituate to single-housing conditions before giving birth.

Once a litter was born, dams and litter were initiated into early life stress through a limited-nesting paradigm or assigned as control (see below). After weaning, male offspring were housed in pairs of two in standard housing conditions as described above. After cannula implantation, male rats were housed individually under the same conditions. All behavioural procedures were performed during the light cycle (10:00 to 15:00). All experiments and procedures were performed in compliance with the European Union Directive 2010/63/EU and approved by the local Institutional Animal Care and Use Committee.

2.2 LIMITED-NESTING PARADIGM

Early life stress (ELS) was induced through a limited-nesting (LN) paradigm adapted from (Ivy, Brunson, Sandman, & Baram, 2008). Parturition was verified every day at 09:00, the day of birth marked as postnatal day (PND) 1. On PND 2, half the dams were assigned randomly to ELS cages and half were assigned to control cages after adjusting the nest size to four females and four males each. All cages received two paper towels that dams used for constructing a nesting area and were not replaced. However, the ELS cages had their nesting material limited through replacement of standard bedding with a steel mesh raised 2.0 cm above the cage floor. Beneath the mesh, two sheets of a paper towel lined the bottom of ELS cages to allow for absorption of urine and droppings. All cages were left completely undisturbed until termination of the LN paradigm on PND 9. On PND 10, all nests were transferred to standard cages until weaning of the male pups at PND 21. Male pups were weighed at the end of the LN paradigm, at weaning, and then every seventh day until surgery.

2.3 MATERNAL BEHAVIOUR SCORING

All cages were observed for maternal behaviour five times each day, from PND 2 until 9. Three observation sessions were conducted during the light phase (10:00, 13:00, and 17:00), and two were conducted during the dark phase (06:30, 20:00). To account for natural variations in activity level throughout the day (lower activity during the light phase compared with higher activity during the dark phase), all analyses were carried out separately for light and dark phases.

During a session, which lasted for 60 minutes, each cage was observed for 5 to 7 seconds at every third minute by an observer in real time. Thus, a total of 20 observations were recorded per session for maternal behaviour which could be characterised as either nursing behaviour or non-nursing behaviour. Nursing behaviour comprised of the dam nursing more than half the litter in high arched (dam visibly arched over the pups and her hind legs extended close to the forelegs), low arched (no obvious arch back or hind leg extension visible), passive nursing (dams on their side) and the dam licking and grooming the pups. Non-nursing behaviour comprised of the dam either being completely outside the nest or in contact with less than half the pups, being in contact with more than half the nest but not nursing, and self-grooming.

An index for quantity of maternal care was calculated across each day of the LN paradigm by summing the number of instances nursing behaviour was recorded per session divided by the total number of observations (20) in an hour. Maternal behaviour was also evaluated for consistency as a measure of the quality of maternal care. For each session, two consecutive instances of observations were compared and a score was given if the maternal behaviour had not altered between them. An index for quality of maternal care was then calculated across each day of the LN paradigm by summing the consistency scores divided by the total number of observations (20) in an hour. Lastly,

total duration in minutes that a dam spent on nursing behaviour in one single stretch, (without any fragmentation of behaviour) was also analysed across all days.

2.4 CORT MEASUREMENTS

At the end of the LN paradigm (PND 10), 30 male pups were removed from their cages at 09:00 and sacrificed through rapid decapitation within two minutes of disturbance. Trunk blood was collected in ice-cold EDTA coated tubes until centrifugation (2,900*g* at 4°C for 15 minutes), plasma was then separated and stored at -80°C until the CORT assay. Plasma corticosterone (CORT) were measured using a commercially available ELISA kit (DetectX Corticosterone Enzyme Immunoassay kit, Arbor Assays, Michigan).

2.5 SURGERY

Rats were anaesthetized with a subcutaneous (s.c.) injection of ketamine (37.5 mg/kg; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion), and surgery was performed according to a standardised protocol (Fornari et al., 2012). Briefly, the rat was positioned in a stereotaxic frame (Kopf Instruments), and two stainless steel guide cannulas (11 mm, 23 gauge; Component Supply Co/SKU Solutions, Fort Meade, FL) were implanted bilaterally with the cannula tips 1.5 mm above the dorsal hippocampus (anteroposterior, −3.4 mm from Bregma; mediolateral, ±1.8 mm from the midline; dorsoventral, 2.7 mm below skull surface; incisor bar, −3.3 mm from interaural) (Paxinos & Watson, 2007). Each cannula was then affixed to the skull with two anchoring screws and dental cement. Stylets (11-mm long 00-insect dissection pins) were inserted into each cannula in order to maintain patency and were removed at the time of infusions. Atipamezole hydrochloride (2.5 mg/kg; Orion) was administered after the surgery for post-anaesthesia recovery and saline (3mL) was administered to prevent dehydration and facilitate renal clearance. Additionally, animals were given the non-steroidal analgesic carprofen (s.c; 4.0 mg/kg body weight; Pfizer) before surgery and 24 hours following for better pain management. Animals were weighed daily and allowed to recover for ten days. During the recovery period, animals were handled three times for 1 minute each to allow them to become accustomed to the infusion process.

2.6 CONTEXTUAL FEAR CONDITIONING

The fear conditioning apparatus (30.5 × 24.1 × 21.0 cm; MED-Associates) was constructed of Plexiglas with aluminium side walls held within sound attenuating wooden cabinets, and located in a brightly lit and isolated room separate from the housing room. The floor of the chamber consisted of a stainless steel grid (19 rods arranged in a straight horizontal plane, 4 mm in diameter, spaced 1.5 cm apart). Rods were wired to a shock source and solid-state grid scrambler (MED-Associates) was used for delivery of foot shock in the training phase, timed and controlled automatically using the Ethovision XT add-on Trial and Hardware Control Module.

All rats were habituated to the training context for 5 minutes without any shock exposure. On the next day, each animal was trained on the contextual fear conditioning (CFC) task. Each training session lasted for 10 minutes and consisted of a two-minute baseline phase, when no foot shock was administered, a training phase when 6 shocks of 1.4 mA, lasting 1 sec were delivered at 1-min intertrial intervals, and a post-training phase when no shock is administered. The chamber was thoroughly cleaned with 70% ethanol between the training of every animal. Twenty-four hours after the training, all animals were returned to the CFC context for 5 minutes; no shock was delivered during this phase.

Additional non-operated ELS and control groups were habituated and trained on the contextual fear conditioning task; however, on the retention test day, the groups were tested for 5 minutes in a different but previously habituated context.

Behaviour was coded twice manually with the aid of a behaviour extracting and coding software (Solomon Coder; http://solomoncoder.com); and analysis was conducted blind to treatment condition. Freezing was defined as a complete cessation of head movements, except movements related to respiration, for more than 1 second. For training the percentage of time rats spent freezing was assessed separately at baseline and then for each interval during consecutive shocks. For CFC test, freezing percentages were calculated for the entire trial.

2.7 PHARMACOLOGICAL MANIPULATION

All pharmacological manipulations were done 1 hour prior to retention testing using freshly prepared drugs.

Corticosterone (CORT; Sigma-Aldrich) was prepared by first dissolving in 100% ethanol and then diluting with 0.9% saline to reach the appropriate concentration. The final concentration of ethanol was 5%. The vehicle for systemic injection contained 5% ethanol in saline only. Animals were either injected with CORT or vehicle (3mg/kg) subcutaneously in the neck region. The dose was based on previous work (P. Atsak, Hauer, et al., 2012), which showed 3mg/kg as the impairing dose of CORT on contextual memory retrieval.

The MAG-L inhibitor, KML-29 (0.2 µg/0.5 µL; Tocris), and CB1 receptor antagonist, AM251 (0.35ng/0.5 µL; Sigma-Aldrich) were prepared by first dissolving in 100% DMSO and subsequently diluting with 0.9% saline and Tween-80 phosphate buffer to reach a final concentration of 2% DMSO and 1% Tween-80. The vehicle for hippocampal infusions contained 5% DMSO, 1% Tween-80 only. Doses were derived from (Atsak et al., 2012; De Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008; Hampson et al., 2011; Morena et al., 2015).

Bilateral infusions of drug or vehicle into the dorsal hippocampus were given by using 30-gauge injection needles connected to 10-µL Hamilton micro syringes by polyethene (PE-20) tubing. The injection needles protruded 1.7 mm beyond the cannula tips, and a 0.5-µL injection volume per hemisphere was infused over a period of 1 minute by an automated syringe pump (Stoelting). The injection needles were retained within the cannulas for an additional 60 seconds to prevent backflow of the drug into the cannulas.

2.8 HISTOLOGY

Rats were anaesthetized with an overdose of sodium pentobarbital (100 mg/kg body weight, i.p.; Sigma-Aldrich) and perfused transcardially with a 0.9% saline solution followed by perfusion with 4% formaldehyde (Merck) dissolved in water (see Appendix C for detailed perfusion protocol). Brains were removed and immersed in 25% sucrose solution in 0.1M Phosphate Buffer for 3 days for cryoprotection. The brains were then frozen using dry ice and 50-µm-thick coronal sections were cut using a cryostat (CM 3050S, Leica Biosystems, Eindhoven, Netherlands). The hippocampal sections were then mounted on gelatin-coated slides and stained with Cresyl fast Violet (Merck). Needle tips were visualised under a light microscope by an observer blind to treatment conditions. Animals were excluded from analysis if the location of needle tip was outside the hippocampus, as determined according to standardised atlas plates (Paxinos & Watson, 2007), or if the animal had extensive hippocampal damage. Approximately, 27 percent of animals were excluded on histology grounds.

2.9 STATISTICAL CONSIDERATIONS

A significance threshold was set at .05, at 95 percent confidence interval prior to data collection. All assumptions for statistical significance testing were run before interpreting the results of each test. Outliers in the data were analysed based on studentized residuals score $z > 3$. All data were assessed for normal distribution using Shapiro-Wilk's test for normality, and violations were reported. Homogeneity of variances and covariances were assessed using Levine's test for equality of variances and Box's M test respectively. Sphericity was analysed using Mauchly's test, *F* statistics were evaluated using Greenhouse-Geisser correction where appropriate. Fisher's LSD was used for all *post hoc* comparisons. Data is presented as mean or mean difference (MD) ± standard error.

Data for maternal behaviour and weight measurements were analysed using two-way mixed model ANOVAs. Analyses were conducted separately for light and dark phases in case of maternal behaviour. Postnatal day on measurement was defined as the within-subject factor, while groups were defined as the between-subject factor. Two sessions of maternal behaviour scoring and one control from weight analysis were excluded based on studentized residuals ($z > 3$).

Data analysis for CORT measurements was conducted using independent-samples t-test. There were no outliers in the data.

A three-way ANOVA (BBW) was conducted for the analysis of changes in freezing behaviour across time (baseline plus 6 time bins) as within-factor, and groups (ELS and control) and later assigned drug treatment as between-subject factors. Planned comparisons were conducted using unpaired t-tests to compare the differences between groups, while planned comparisons using paired t-tests were performed to compare the differences between time bins.

For retention testing, all analysis was performed using two-way ANOVA and *post hoc* tests were run for each simple main effects and main effects separately.

Chapter 3 Results

3.1 LIMITED-NESTING PARADIGM ALTERS THE QUANTITY AND QUALITY OF MATERNAL CARE

3.1.1 Reduced maternal care

To investigate how limiting availability of nesting materials alters the overall quantity of nursing interactions between dam and pups, a comparison of pooled scores from all the nursing interactions across each day was conducted between LN and control cages for light and dark phases. Results from two-way mixed model ANOVA (light phase) were significant for group differences in quantity of care provided to the litter ($F_{1, 60}$ = 30.47, p < .01) (figure 1). In addition, there was an interaction between groups and postnatal day on the quantity of maternal care ($F_{5.1, 304}$ =3.30, p < .01, ε = .72). However, no effect of post-natal day ($F_{5.1, 304}$ = 1.63, p = 1.6, ε =.72). Post hoc pairwise comparisons indicate that for the light phases, dams in LN cages engaged in significantly lesser nursing interactions with their pups across all treatment days except the last (all p < .02).

No significant differences were present in the quantity of maternal care between groups across dark phase of measurement days (all p > .10).

3.1.2 Fragmented maternal care

To assess if LN paradigm induces a fragmentation and unpredictability of maternal care, analysis for consistency of behaviour was conducted between dams in LN and control cages using a two-way mixed model ANOVA. Results from two-way mixed model ANOVA conducted consistency measurements for light phases indicate a statistically significant effect of group on the consistency of care across the length of the LN treatment ($F_{1, 60}$ = 11.19, p < .01) (figure 2). There was also an effect of postnatal days on the measure of consistency ($F_{5.5, 329}$ = 5.14, p < .01, ε = .79). However, no interaction was found between groups and postnatal day on the consistency of maternal care ($F_{5.5, 329}$ = 1.43, p = .21, ε = .79). Post hoc analysis revealed that consistency of maternal interactions was significantly affected by the limited availability of nesting material during the initial three days of the treatment, i.e. PND 2, PND 3 and PND 4, as assessed by t-test for significance (p <.05). Differences in the inconsistency of maternal care were found only for dam-pup interactions during the light phase.

3.1.3 Duration of nursing epoch

A two-way mixed model ANOVA was conducted to determine whether there was a difference in mean duration of a nursing epochs (defined as one instance of dam-pup interaction without any alteration in behaviour) between LN and control cages.

Results from the analysis revealed a statistically significant group difference between LN and control cages in the mean duration of time a dam spent nursing their pups across days (group differences in quantity of care provided to the litter ($F_{1, 52}$ = 16.22, p < .01) (figure 3). There was also an effect of postnatal days on the duration of nursing epochs ($F_{1, 52}$ = 3.19, p < .01). However, no interaction was present between groups and postnatal day on the mean duration of a nursing epoch ($F_{1, 52}$ = 1.97, p = .06). Post hoc pairwise comparisons indicated that for the light phases, dams in LN cages engaged in significantly lesser nursing interactions with their pups across all treatment days except the last (all p < .02).

Taken together, these findings suggest that LN conditions adversely affect both the quantity and quality of maternal interactions with pups.

3.2 ALTERED MATERNAL BEHAVIOUR INDUCES CHRONIC EARLY LIFE STRESS IN PUPS

3.2.1 Altered corticosterone levels

To assess if our model of fragmented and unpredictable maternal care alters CORT levels in ELS pups, an independent-samples t-test was run to determine if there were differences in CORT levels between ELS and control groups at the termination of the LN paradigm. A

statistically significant difference (p = .02) was found in mean CORT concentrations between the two groups with ELS pups having an elevated plasma CORT levels (6.1 ± 0.6 ng/mL) at the of the LN paradigm compared to control pups (4.1 ± 0.6 ng/mL) (figure 4).

3.2.2 Persistent effects on development

To investigate how ELS affects weight gain during development, all male rats were weighed once at the end of the limited nesting paradigm and then every 7 days until surgery. A two-way mixed ANOVA revealed a statistically significant effect of group on mean weight across the measurement days and postnatal day on weight ($F_{1, 178}$ = 14.02, p < .01), as well as a statistically significant effect of time on weight gain ($F_{1.28, 228}$ = 10,908.51, p < .01, ε = .214). There was also a significant interaction between groups and post-natal day on weight gain ($F_{1.28, 228}$ = 6.06, p = .01, ε = .214), suggesting that the rate of weight gain was significantly different for the ELS and control groups.

Orthogonal tests for differences between ELS and control animals across each postnatal period confirmed that controls had a significantly higher mean weight gain at each measurement in time (all p < .01) (figure 5).

3.3 EARLY LIFE STRESS MODULATES THE EFFECT OF CORTICOSTERONE, BUT NOT MAGL INHIBITOR, ON IMPAIRMENT OF CONTEXTUAL FEAR MEMORY RETRIEVAL

The overarching aim of the experiment was to investigate whether corticosterone suppresses fear memory retrieval in adult rats with a history of ELS, and if a direct augmentation of the 2-AG response can lead to a suppression of contextual memory retrieval in these rats.

To this end, four separate treatment groups were tested in contextual fear conditioning one hour after pharmacological manipulations. One group received systemic injection of CORT (3 mg/kg s.c.) together with bilateral hippocampal vehicle infusion, another group received vehicle injections systemically and together with bilateral hippocampal infusions of the MagL inhibitor KML-29 (0.2 µg in 0.5 µL), a third group was given systemic vehicle injection with bilateral infusions of KML-29 together with CB1 antagonist AM251 (0.2 µg and 0.35 ng in 0.5 µL). The control treatment group did not receive any drug either systemically or through direct hippocampal infusions.

3.3.1 Training

Three-way mixed ANOVA for freezing scores during training confirmed that ELS and control groups did not differ in the acquisition of fear memory ($F_{1, 112}$ = 2.09, p = .15). There were also no differences in acquisition rates between later assigned drug groups ($F_{3, 112}$ = .54, p = .66). Indeed, all groups acquired contextual fear conditioning task as indicated by the statistically significant rate of increase in freezing percentage at each interval ($F_{4.54, 508}$ = 318.7, p < .01, ε = .756).

3.3.2 Retention test

A two-way ANOVA revealed statistically significant main effect of treatment on the mean time rats spent freezing to the context ($F_{3,78}$ = 2.98, p = .04). However, the main effect of groups did not reach significance ($F_{1, 78}$ = .08, p = .77). There was also no significant interaction between groups and treatments ($F_{3, 78}$ = 1.63, p = .19).

As shown in Figure 6, pairwise comparisons indicated that ELS and control groups treated with vehicle only did not differ in freezing to the context during retention trial (p = .77).

Administration of CORT 1 hour prior to retention testing had a significantly different modulation effect on freezing levels between early life stressed animals and controls (main effect of group on CORT treatment, ($F_{1, 78}$ = 4.22, p = .04). Pairwise comparisons revealed that CORT administration to control rats significantly impaired freezing scores when compared to vehicle-treated controls (MD = 24.7 ± 9.5; p = .01). However, CORT administration to ELS rats did not significantly reduce freezing scores relative to vehicle-treated ELS rats (p = .84).

Direct augmentation of 2-AG in the hippocampus using KML-29 1 hour prior to retention testing reduced mean freezing response across groups as compared to vehicle (MD = 19.7 ± 7.0, p < .01). In addition, no differences were found between KML-29 treated ELS animals and controls in freezing during retention testing ($F_{1,78}$ = .31, p = .58). Additionally, there was a statistically significant reduction in the mean freezing response during retention testing in KML-29 treated ELS when compared with CORT-treated (MD = 19.5 ± 9.9, p = .05), as well as vehicle-treated ELS animals (MD = 21.9 ± 9.5, p = .05).

To assess whether the KML-29 effect described above is due to an interaction of 2-AG with CB1 receptors within the hippocampus, we analysed the different freezing responses during retention trials between infusion of KML-29 alone and with an infusion of KML-29 concurrently with the CB1 receptor antagonist AM251. A trend driving an increase in freezing rates was found across groups when 2-AG

availability is increased while blocking CB1 concurrently (MD = 12.1 ± 7.3, p = .10). Moreover, there were no statistically significant differences in freezing during retention in animals treated with KML-29 concurrently with AM251 and those treated with vehicle alone (MD = 8.0 ± 6.6, p= .23). No significant differences were found in KML-29+AM251 treatment groups between early life stressed and control groups (MD = 6.0 ± 9.9, p = .60).

These results indicate that CORT treatment did not impair retrieval in ELS animals. Whereas, directly augmenting the availability of hippocampal 2-AG had an impairing effect on retrieval in both ELS and control animals.

Lastly, to examine the possibility that CORT treatment directly influences the expression of freezing in control or early life stressed animals, a separate cohort of animals was trained on the contextual fear conditioning paradigm. 24 hours later, the animals were administered CORT systemically (3mg/kg), and 1 hour later, tested in a non-training context to which they were previously habituated to. As shown in figure 7, ELS treatment did not differentially modulate the effect of CORT on the expression of freezing behaviour to a non-training context ($F_{1, 34}$ = 1.43, p = .24). No main effect of CORT ($F_{1, 34}$ = .10, p = .75), or an interaction of CORT with groups was found to be significant either ($F_{1, 34}$ < .01, p = .97). These findings indicate that neither early life stress treatment nor CORT administration directly altered the expression of freezing behaviour to the non-training context in animals.

Chapter 4 Figures

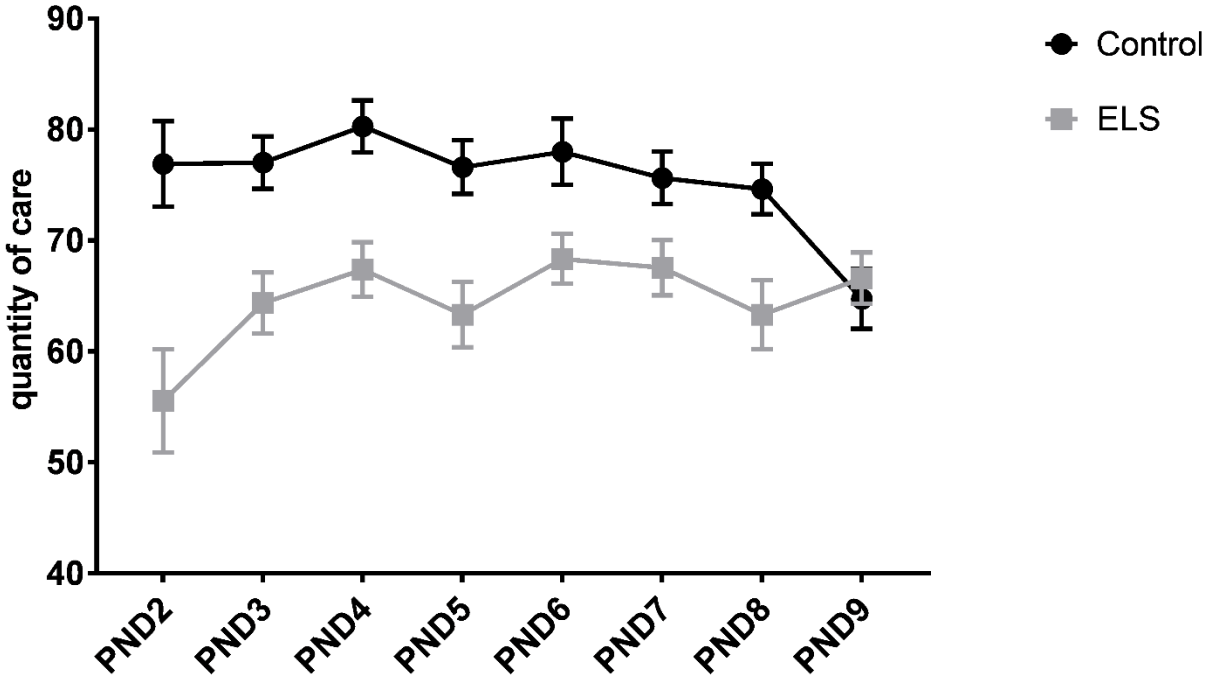

Figure 1 Limited-nesting paradigm reduces the quantity of care received by the pups in ELS cages.

Graphic depiction of the average time dams spend nursing their nest throughout PND 2- PND 8 during the light phase. Dams in limited nesting cages spend significantly lower time nursing their pups when compared to controls ($F_{1,60} = 30.47$; $P < .01$, effect of group, two-way mixed model ANOVA). Data represents mean percentage of time ± SEM.

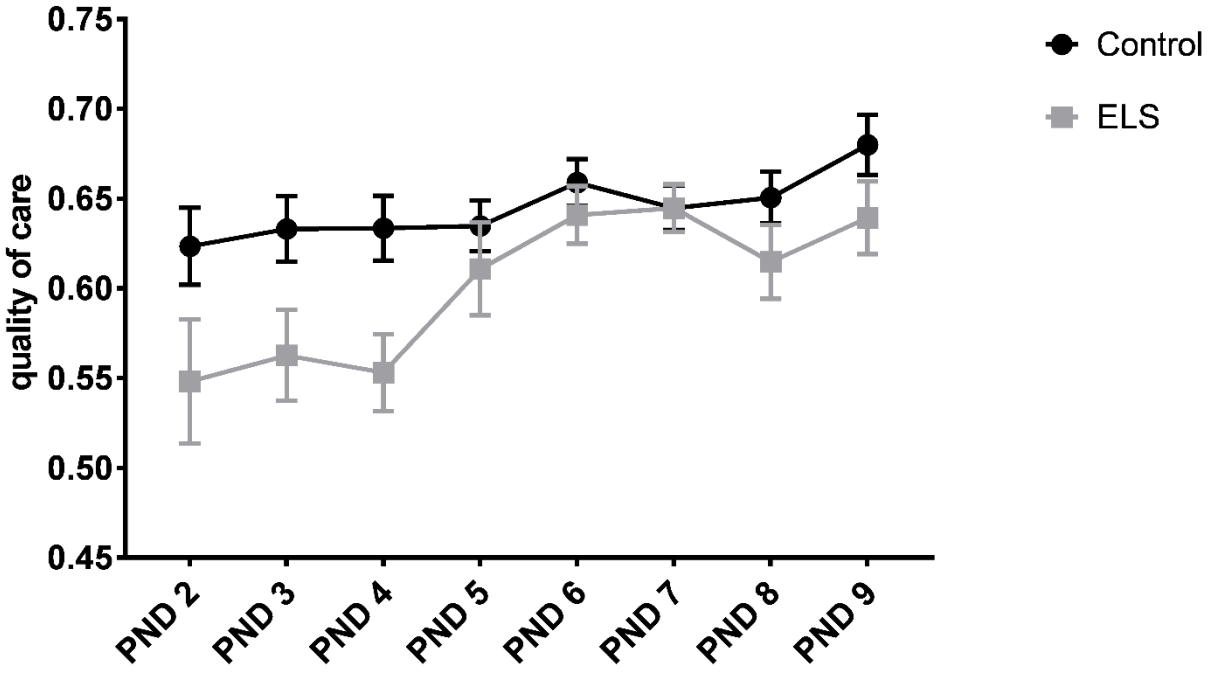

Figure 2 Limited-nesting paradigm induces fragmentation and unpredictability in the care received by pups in ELS cages

Graphic depiction of the quality of care provided by dams throughout PND 2- PND 8 during the light phase. The quality of maternal care, measured as consistency index, was significantly lower for dams in ELS cages compared with controls ($F_{1,60}$ = 11.19; P < .01, effect of group, two-way mixed model ANOVA). Data represents mean consistency index ± SEM.

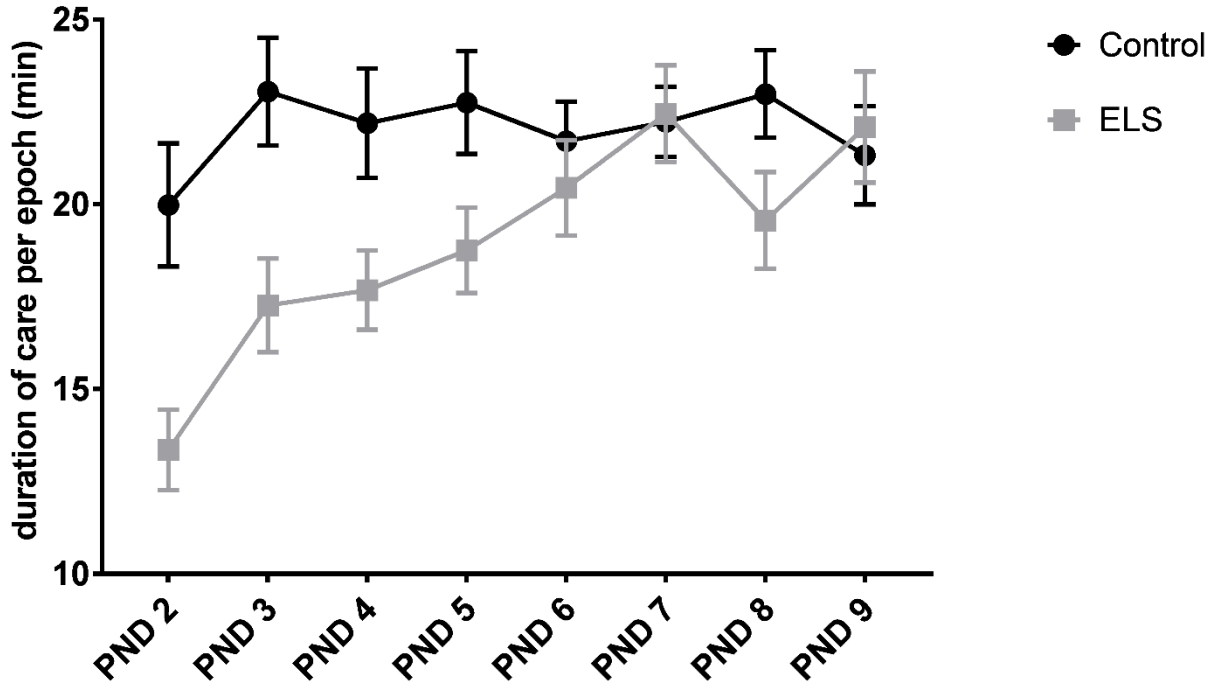

Figure 3 Limited-nesting paradigm reduces the mean duration time dams spent nursing their pups in one epoch

Graphic depiction of the mean duration of time dams spent nursing before altering their behaviour; 2- PND 8 during the light phase. The dams in LN cages spent much less time providing nursing care to their pups before altering their behaviour than did controls ($F_{1,60} = 16.22$; $P < .01$, effect of group, two-way mixed model ANOVA). Data represents mean duration of time in minutes ± SEM.

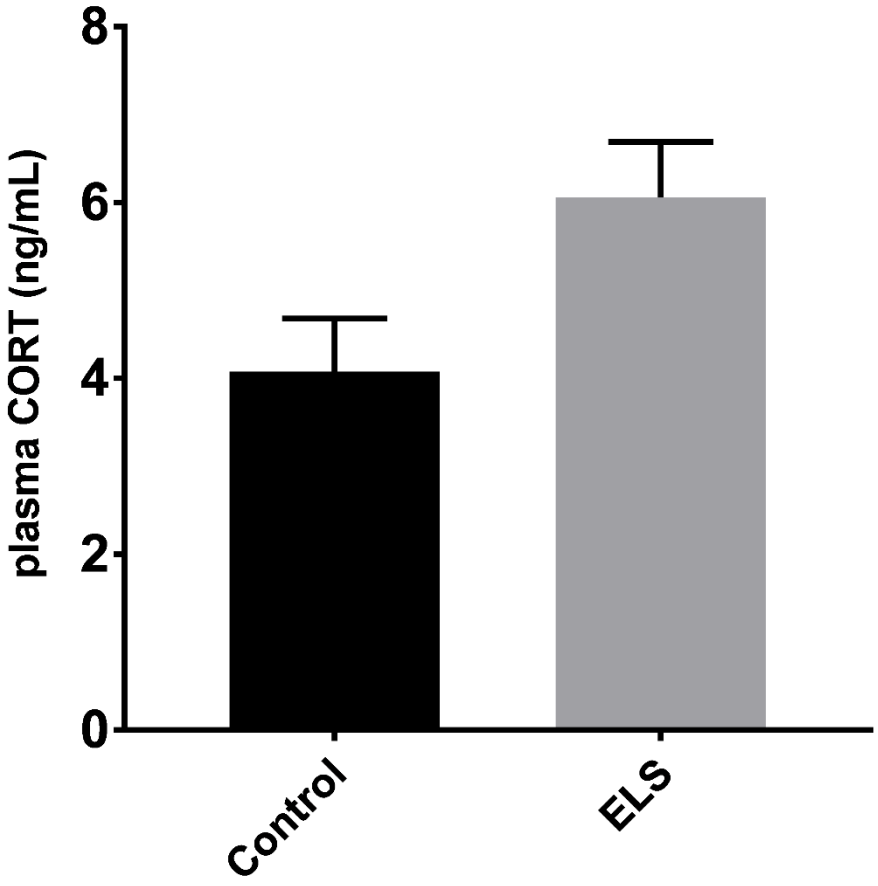

Figure 4 Early life stress causes an elevated plasma corticosterone concentration in pups at the end of the limited-nesting paradigm

Plasma corticosterone (CORT) was measured using ELISA. CORT was significantly higher in the ELS pups compared with controls. Trunk blood for plasma analysis was collected in morning of PND 10, after rapid decapitation. ($p = 0.02$, independent sample t-test). Data represents mean levels of plasma CORT as ng/ml ± SEM.

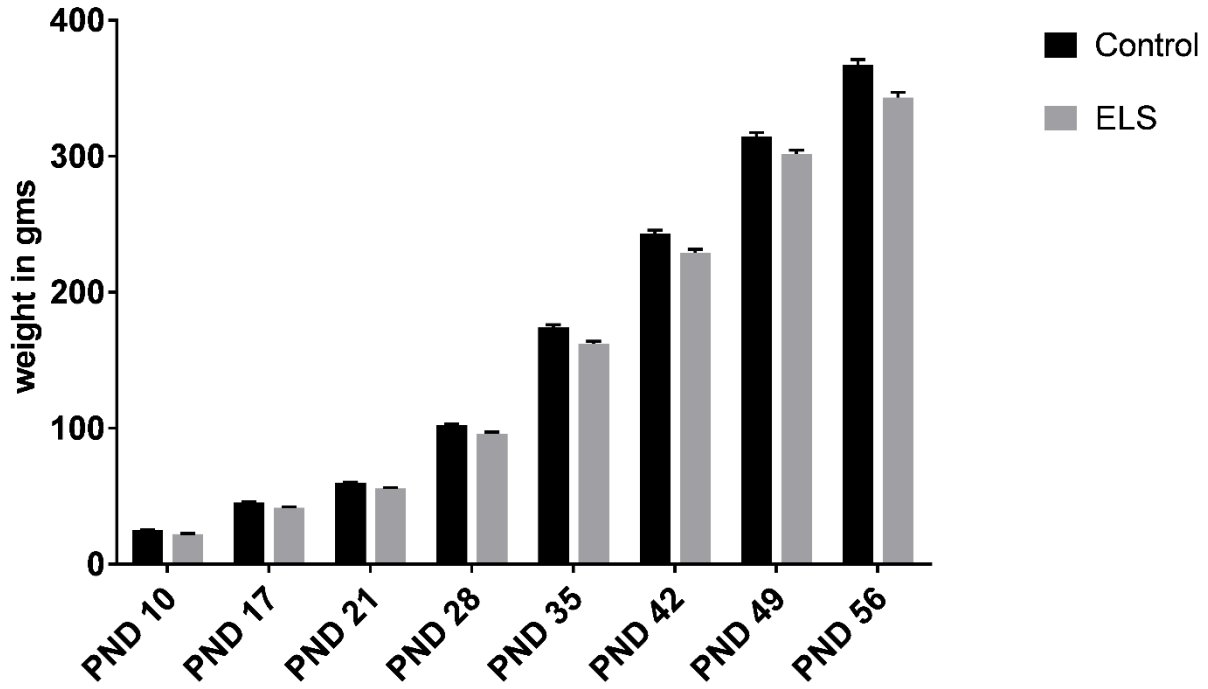

Figure 5 Early life stress causes persistently lower weight in developing rats

Graphic depiction of the mean weight of pups across PND 10 until PND 56 (week 7). Pups with early life stress have a retarded growth when compared with controls across all measurement periods ($F_{1,178} = 14.02$; $P < .01$, effect of group, two-way mixed model ANOVA). Data represents mean weight in grams ± SEM.

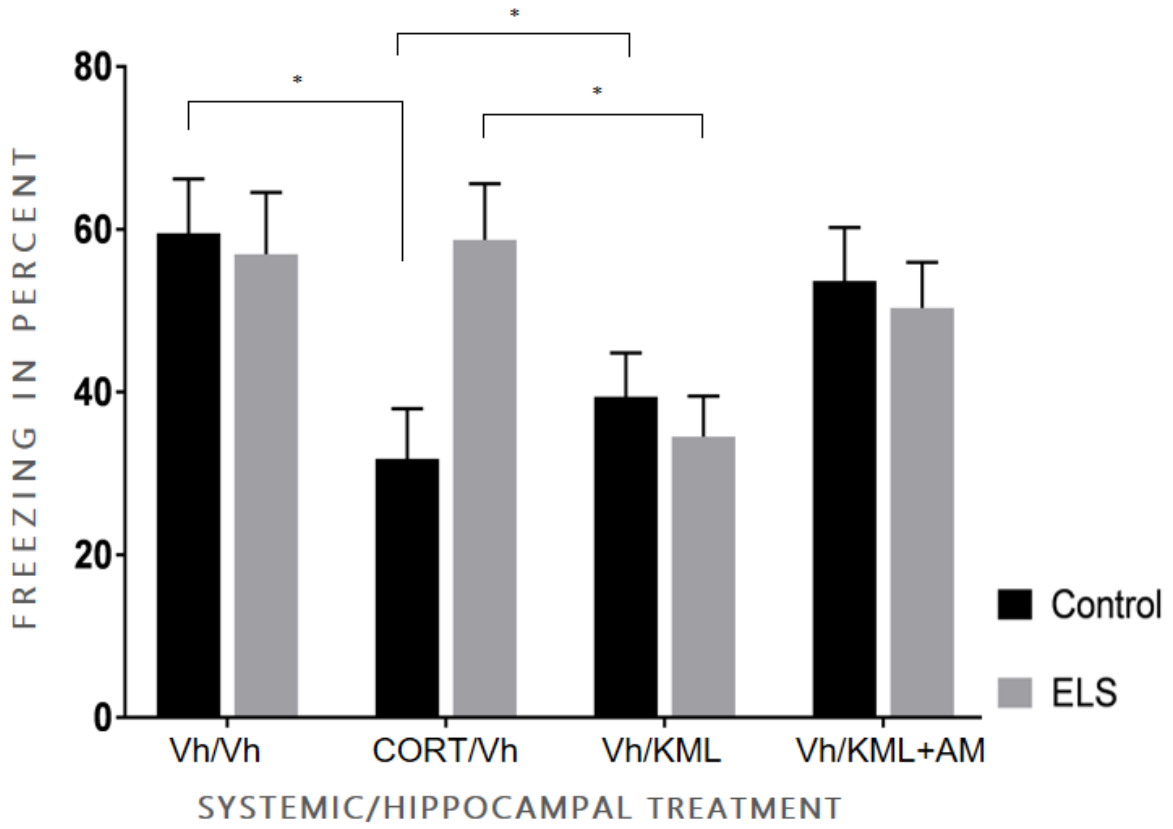

Figure 6 Early life stress modulates the effect of corticosterone, but not of MagL inhibitor, on impairment of contextual fear memory retrieval

Data from contextual fear retention testing. All pharmacological manipulations were done one hour before retention testing. CORT was injected systemically (3mg/kg; s.c.). Whereas KML-29 (0.2 µg/0.5 µL) and AM251 (0.35ng/0.5 µL) were given via bilateral hippocampal infusions. In ELS rats, CORT does not impair retrieval when compared with rats treated only with vehicle. However, in control rats a systemic injection of CORT significantly impairs retrieval when compared with rats treated only with vehicle. Bilateral hippocampal infusion of KML-29 significantly impairs retrieval in both ELS and control rats when compared with rats treated with vehicle only. In both ELS and control rats, no significant differences were present between rats treated with vehicle only and those treated with a bilateral infusion of KML-29 together with AM251. Data represents mean freezing percentage ± SEM. Significance tests done using Fisher's LSD ($p < .01$).

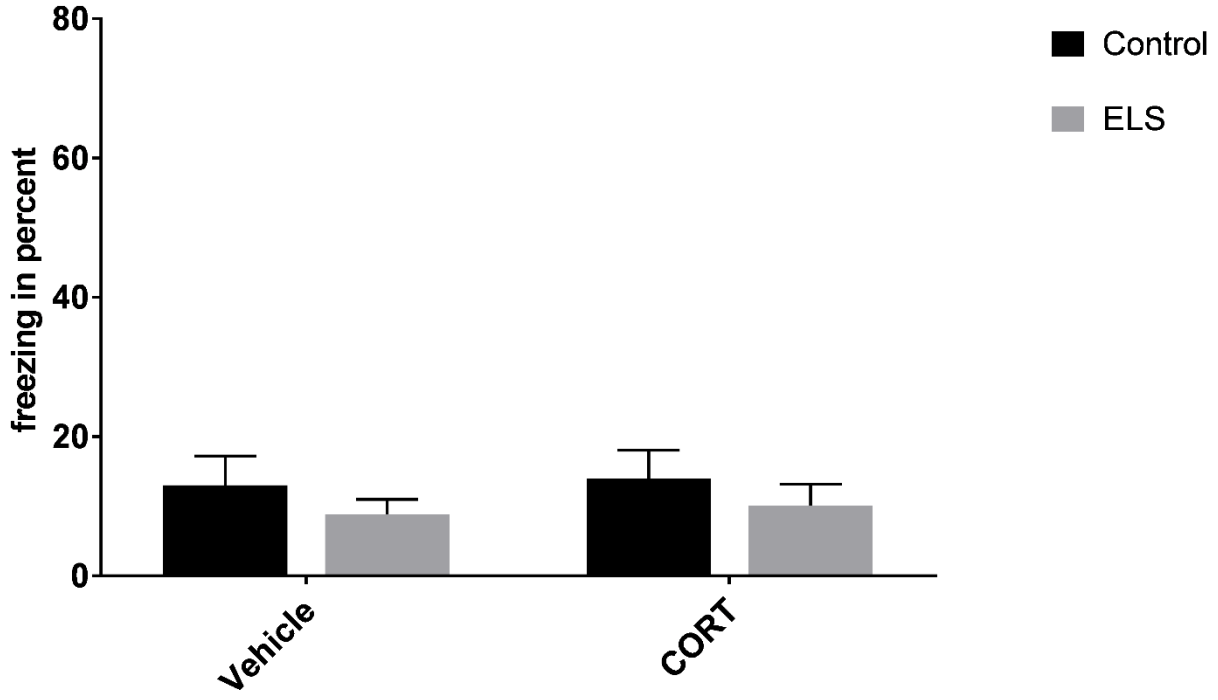

Figure 7 History of early life stress and treatment with CORT do not directly influence freezing in non-training context in both ELS and control animals

Data from control group tested in a non-training, but previously habituated, context. All animals were given CORT systemically (3mg/kg s.c.). There were no differences between ELS and control groups on the expression of freezing behaviour to a non-training context ($F_{1, 34}$ = 1.43, p = .24). The effect of CORT was not found to be significantly different across groups either ($F_{1, 34}$ = .10, p = .75). There was also no interaction effect between the two ($F_{1, 34}$ < .01, p = .97).

Chapter 5 Discussion

There is substantial evidence that the endocannabinoid system, in concert with glucocorticoids, plays a critical role in the maintenance of a healthy stress response system which is capable of adapting to the environment (Morena, Patel, Bains, & Hill, 2016). In addition, this system is especially vulnerable to stress during critical developmental periods (Marco et al., 2014). Therefore, it is very important to understand the link between ELS and the later development of vulnerabilities to stress-induced neurocognitive disorders, especially those that underlie an aberrant modulation of emotional memory under stress. The basis for our study came from previous work by Atsak et al. (2012), which provided evidence that glucocorticoid exerts its effect on contextual fear memory retrieval, through an interaction with 2-AG acting on hippocampal CB1 receptors.

Briefly, we confirmed our hypothesis that in ELS rats, a systemic injection of CORT fails to impair retrieval of contextual fear memory, whereas a direct augmentation of the 2-AG response in the hippocampus is sufficient to suppress memory retrieval in both ELS rats and controls. In addition, we replicated results from other studies employing an LN model for chronic ELS induction and validated the paradigm for future studies in our lab. To the best of our knowledge, this is the first study to investigate the link between ELS-induced persistent alterations in endocannabinoid signalling and the resultant dysregulations in glucocorticoid modulation of traumatic memory retrieval.

In our experiment, no baseline differences were present between vehicle-treated ELS rats and controls in contextual fear response during retention testing. Indeed, both ELS rats and controls that were treated with vehicle systemically, as well as locally within the hippocampus, showed robust retention of contextual memory (50% freezing scores). This is consistent with freezing scores seen in the previous study (P. Atsak, Hauer, et al., 2012). While the exposure to stress during early life has an effect on the developmental trajectory of fear retention system and results in longer retention of fear memories across time, other studies also reported no differences in fear memory retention between ELS and control animals tested 24 hours after training (Callaghan & Richardson, 2012; Gale et al., 2004). Although these studies were conducted using maternal separation, as opposed to LN paradigm, there is evidence that the early life effects on retention memory system were mediated through CORT. Thus, maternal separation and LN paradigm should have similar effects on fear memory retention.

In addition, we found neither any differences in the acquisition rates of contextual fear memory between ELS rats and controls nor later treatment conditions. Indeed, several studies have shown that neither a history of early life trauma nor an impaired endocannabinoid system causes a difference in the acquisition of fear memory in adulthood (Chocyk et al., 2014; Marsicano et al., 2002; Suzuki et al., 2004). Thus, it can be concluded that the administration of CORT prior to retention testing selectively modulated the retrieval of hippocampus-dependent contextual fear memory in both ELS and control animals (Cai, Blundell, Han, Greene, & Powell, 2006; Schutsky, Ouyang, Castelino, Zhang, & Thomas, 2011).

As predicted, our findings indicate that systemic CORT administration does not impair retrieval of contextual fear memory in rats with an ELS history; whereas controls treated with CORT had a robust suppression of contextual fear memory retrieval. Conversely, direct hippocampal infusions of KML-29 1 hour prior to retention testing impaired retrieval of contextual fear memory in both ELS and controls. KML-29 is a potent and highly selective MAGL inhibitor (Chang et al., 2012). Presynaptic MAGL is known to hydrolyse 2-AG released from activated postsynaptic neurones and prevent accumulation of 2-AG around presynaptic terminals. By inhibiting the activity of this enzyme, retrograde 2-AG signalling within the hippocampus can be augmented (Hashimotodani, Ohno-Shosaku, & Kano, 2007). Thus, a direct hippocampal infusion of KML-29 prior to retention testing likely increased 2-AG levels in both ELS and control rats. Since CORT exerts its effect on modulation of contextual fear memory retrieval via hippocampal 2-AG, an augmentation of the 2-AG response in hippocampus directly impaired the retrieval of contextual fear memory in both ELS rats and controls. Together, these results provide compelling evidence that the inability of CORT to suppress retrieval in ELS rats arises from a failure of ELS rats to mount an adequate 2-AG response to acute stress.

We also provide evidence that the modulatory effect of hippocampal 2-AG on contextual fear memory retrieval is dependent on the CB1 receptor. These findings are in line with previous work which showed that CORT exerts its effect on contextual fear memory retrieval through an interaction with endocannabinoids acting via a CB1 receptor-dependent mechanism (P. Atsak, Hauer, et al., 2012). Traditionally, the neurocognitive effects of endocannabinoids have been reported to function through the CB1 receptors; however, accumulating evidence points to a possible involvement of the CB2 receptor in central nervous system as well. (Atwood, Straiker, & Mackie, 2012; Van Sickle et al., 2005). These CB2 receptors may even be involved in the anxiolytic-like responses induced by 2-AG; although these effects are reported to occur in the absence of alterations in cognitive functions (Busquets-Garcia et al., 2011). Very recently, a role of CB2 receptors in modulating the excitation threshold of nerve cells in the hippocampus has also been reported (Stempel et al., 2016). Future

studies may shed further light on the differences in neurocognitive functions of CB1 and CB2 receptors, as possible changes in functioning of these receptors as a result of ELS.

One caveat of our study is that we did not directly explore the possibility that an augmentation of hippocampal 2-AG might have an effect on the expression of freezing behaviour in a context-independent manner. There have been studies that implicate 2-AG as having a regulatory effect on anxiety through an action on CB receptors (Busquets-Garcia et al., 2011; Sciolino, Zhou, & Hohmann, 2011; Sumislawski, Ramikie, & Patel, 2011). However, various studies have reported hippocampal infusions of cannabinoid agonists to impair spatial memory without directly affecting the expression of behaviours that were assessed as an index of memory (Egashira, Mishima, Iwasaki, & Fujiwara, 2002; Lichtman, Dimen, & Martin, 1995; Wegener, Kuhnert, Thüns, Roese, & Koch, 2008). Moreover, the anxiolytic effect of 2-AG is reported to be mediated dominantly via CB1 receptors expressed on glutamatergic neurones (Rey, Purrio, Viveros, & Lutz, 2012; Ruehle et al., 2013). Whereas, in the model we have reported previously, glucocorticoid modulation of emotional memory retrieval is mediated via the action of 2-AG on CB1 receptors located on GABAergic interneurons in the hippocampus.

According to this model, glucocorticoids recruit the endocannabinoid system by first binding to a membrane-bound GR, which activates the intracellular cAMP/PKA signalling cascade to induce endocannabinoid synthesis and release. The endocannabinoids then bind to presynaptic CB1 receptors on GABAergic terminals, which results in a rapid suppression of GABAergic signal. This suppression of GABAergic transmission leads to a disinhibition of norepinephrine release, ultimately resulting in the modulation of emotionally arousing memories (P. Atsak, Roozendaal, et al., 2012). Very recently, this model was validated in a study that employed in-vitro electrophysiological approach on neurones of the basolateral amygdala (Di et al., 2016). However, future studies are required to shed more light on the exact mechanisms through which trauma during a critical period in development leads to a persistent alteration of the 2-AG response to acute stress.

The implications of our findings on ELS-induced dysmodulation of emotional memory retrieval are manifold. First, the finding that CORT does not impair retrieval in adult rats with a history of ELS is an important link between early trauma and later development of neurocognitive dysfunctions. Glucocorticoids play an essential role in the reduction of aversive memory retrieval. In the absence of this impairment, excessive retrieval causes re-experiencing, and thus, reconsolidation of aversive memory traces (D. J.-F. de Quervain & Margraf, 2008; Soravia et al., 2006). Therefore, a dysfunctional glucocorticoid modulation of traumatic memory recall is an important factor for later development of stress-induced psychopathologies, such as anxiety-related disorders and PTSD. Another mechanism through which ELS may play a maladaptive role is by altering the endocannabinoid regulation of stress response system. It has been reported that the tonic AEA under steady state condition keeps the HPA axis constrained (Gray et al., 2015; Patel, Roelke, Rademacher, & Hillard, 2005), while the phasic 2-AG response acts to bring about the termination of stress response (Dallman, 2005; Diorio, Viau, & Meaney, 1993). In this manner, 2-AG plays a critical role in feedback regulation of the stress response system and maintenance of allostasis. Thus the absence of a 2-AG response to acute stress in ELS animals may underlie a maladaptive stress system and development of a vulnerable phenotype. Yet another mechanism through which endocannabinoids, particularly 2-AG, are protective is through curbing some of the harmful effects of chronic stress on neural plasticity. Recently, it was reported that an augmentation of 2-AG through MAGL prevents impairments in hippocampal neurogenesis resulting from exposure to chronic stress (Zhang et al., 2015). Moreover, augmentation of 2-AG signalling may even be able to overcome the desensitisation and resultant loss of CB1 receptor signalling after chronic stress (Patel, Kingsley, Mackie, Marnett, & Winder, 2009; Sumislawski et al., 2011). Considering the importance of 2-AG acting through CB1 receptors in buffering against the effects of stress, ELS-induced impairments in this system may be an important risk factor for later development of stress-induced disorders such as anxiety and PTSD. To conclude, our results provide compelling evidence for further investigation in the role of altered endocannabinoid system in the development of later neurocognitive pathologies in adults with a history of ELS.

In addition to elucidating the role of persistent alterations in endocannabinoid system from trauma in early life, we also validated the LN paradigm for induction of chronic ELS in pups. Dams in our LN cages not only provided a decreased amount, but also a highly fragmented and erratic quality, of nursing care to their nest. These results are in line with studies that have employed the LN paradigm in both mice and rats (Ivy et al., 2008; Rice, Sandman, Lenjavi, & Baram, 2008). In our study, the maternal behaviour of LN dams was especially inconsistent when compared to the behaviour of control dams during the first 4 days of the experiment. This is in line with the study by Ivy et al., where the biggest differences in consistency of nurturing behaviour were observed in the first 4 days of the ELS paradigm (Ivy et al., 2008). The analysis of consistency in our study as well as the studies by Ivy et al., (2008) and Rice et al., (2008) did not account for fragmentation of nursing behaviour differentially; that is to say that a dam away from its nest throughout the observation session would be as consistent as a dam nursing its nest throughout the observation period. However, differences in mean duration of nursing epochs between LN and control cages suggest that dams in LN cages spent much less time providing nursing care to their pups before altering their behaviour than did controls; these differences could be observed until PND 8. This observation can also be interpreted as higher fragmentation in the nursing behaviour of dams in LN cages compared with that of control.

The advantage of employing LN paradigm, instead of the more widely used maternal separation model, for induction of ELS, is that stress in LN paradigm is more of a chronic unpredictable and uncontrollable nature, instead of being intermittent and transient (Molet, Maras, Avishai-Eliner, & Baram, 2014). Moreover, stress resulting from LN paradigm is a closer reflection of human studies related to neglect and abuse early in life -where the caregiver's interactions with the child are present albeit erratic (Bennice & Resick, 2003; Sousa et al., 2011).

In addition to altering the maternal interaction with pups, the LN paradigm also led to an increased plasma CORT level in ELS pups compared to controls at the end of the LN paradigm. ELS models based on maternal separation have widely been reported to alter basal CORT levels and related neuroendocrine responses (Levine, Huchton, Wiener, & Rosenfeld, 1991; Moriceau & Sullivan, 2006). However, it is also reported that pups can learn to predict the separation cycle with basal CORT levels no longer elevated (Daskalakis et al., 2011). It has been shown that a quantitative reduction of maternal care alone is not sufficient by itself to alter the stress response system in the offspring (Macrí, Mason, & Würbel, 2004). Therefore, it is likely the inconsistency and unpredictability of maternal care together with the reduced quantity elicited by LN paradigm in our study, which constituted a source of ongoing, non-habituating stress and resulted in the observed changes in plasma corticosterone levels in ELS pups (Brunson, 2005; Gilles, Schultz, & Baram, 1996).

ELS pups in our study also had lower weight at the end of the LN paradigm compared to controls. Moreover, the rate of growth remained disproportionately lower throughout the weeks leading up to behavioural tests. Similar results were observed in other studies employing LN paradigm for induction of early life trauma (Gilles et al., 1996; Maniam, Antoniadis, Wang, & Morris, 2015; Rice et al., 2008). Taken together, these results suggest that LN paradigm elicited aberrations in normal, healthy dam-pup interactions, and resulted in a robust induction of chronic ELS in pups.

In summary, the present findings indicate that ELS induces persistent alterations in the hippocampal 2-AG system and lead to altered modulation of emotional memory retrieval under acute stress. This revelation can lead to a better understanding of ELS-induced vulnerabilities to psychiatric disorders and altered neurocognitive phenotypes. Future studies should focus on elucidating the exact mechanisms through which ELS induces an alteration in the endocannabinoid response to stress. At least one recent study provides first evidence that the effects of early stress on neurocognitive performance may be reversed in adulthood (Alteba, Korem, & Akirav, 2016). However, more studies are needed in the future that look to identify possible developmental window in which persistent effects of ELS on the endocannabinoid system could be rescued. Moreover, memory retrieval is just one system through which glucocorticoids modulate memory under stress Further studies are required to study the consequences of ELS on other memory systems which are also modulated by glucocorticoids. In addition, studies should be expanded to compare the effects of trauma in both early life and adolescence, since both periods are known to be sensitive to the long-term programming effects of stress (Andersen & Teicher, 2008; Casey et al., 2010; Paus, Keshavan, & Giedd, 2008). Moreover, stress may reprogram the brain differently depending on the developmental period (Cordero, Just, Poirier, & Sandi, 2016). Additionally, some studies have reported sex differences in the effects of ELS on both cognition and the endocannabinoid system (Bale & Epperson, 2015; Loi, Koricka, Lucassen, & Joëls, 2014; Viveros et al., 2012). These differences in sex warrant further investigation since they are also present in the epidemiological literature reporting on stress-induced disorders (Bangasser & Valentino, 2014).

Chapter 6 References

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