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Early Life Stress Alters the Developmental Trajectory of Corticolimbic Endocannabinoid Signaling in Male Rats

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Abstract

Early-life stress modulates the development of cortico-limbic circuits and increases vulnerability to adult psychopathology. Given the important stressbuffering role of endocannabinoid (eCB) signaling, we performed a comprehensive investigation of the developmental trajectory of the eCB system and the impact of exposure to early life stress induced by repeated maternal separation (MS; 3 hours/day) from postnatal day 2 (PND2) to PND12. Tissue levels of the eCB molecules anandamide (AEA) and 2-arachidonoylglycerol (2-AG) were measured after MS exposures, as well under basal conditions at juvenile (PND14), adolescent (PND40) and adult (PND70) timepoints in the prefrontal cortex (PFC), amygdala and hippocampus. We also examined the effects of MS on CB_1 receptor binding in these three brain regions at PND40 and PND70. AEA content was found to increase from PND2 into adulthood in a linear manner across all brain regions, while 2-AG was found to exhibit a transient spike during the juvenile period (PND12-14) within the amygdala and PFC, but increased in a linear manner across development in the hippocampus. Exposure to MS resulted in bidirectional changes in AEA and 2-AG tissue levels within the amygdala and hippocampus and produced a sustained reduction in eCB function in the hippocampus at adulthood. CB_1 receptor densities across all brain regions were generally found to be downregulated later in life following exposure to MS. Collectively, these data demonstrate that early life stress can alter the normative ontogeny of the eCB system, resulting in a sustained deficit in eCB function, particularly within the hippocampus, in adulthood.

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Introduction

It is widely accepted that early life stress exposure can have long-term ramifications for the development and maturation of limbic circuits that subserve the regulation of stress, mood and anxiety (Andersen, 2015; Callaghan et al., 2014; Gee et al., 2013; Malter Cohen et al., 2013). In fact, several clinical studies have identified that exposure to early life stress is one of the biggest predictors of vulnerability to the development of psychiatric illnesses, such as major depression and post-traumatic stress disorder (PTSD), in adulthood (Davidson & McEwen, 2012; Heim & Binder, 2012; Pratchett & Yehuda, 2011). Interestingly, while exposure to stress during the early life period clearly has immediate effects on the activation of stress responsive systems in the brain and body, many of these detrimental effects do not emerge until later in life, such as during adolescence or even adulthood (Andersen, 2015; Gee & Casey, 2015). A large body of evidence has focused on the potential role of epigenetic programming in these delayed effects, with a particular focus on the effects of early life stress on the methylation and silencing of glucocorticoid receptor expression (Klengel & Binder, 2015; Turecki & Meaney, 2016). This stream of research suggests that early life stress produces a long-lasting suppression of glucocorticoid receptor expression, particularly in the hippocampus, which results in a state of glucocorticoid resistance and impaired regulation of the neurobiological cascades that are evoked by stress exposure in adulthood (Turecki & Meaney, 2016; Zhang et al., 2013). While these findings are quite promising and provide a logical mechanism for the transfer of stress in early life to behavioral alterations as the animal matures, the investigation of other target systems that could result in altered stress regulation in adulthood are warranted at this early stage.

The endocannabinoid (eCB) system is a neuromodulatory lipid signaling system in the brain that primarily acts as a retrograde signaling system that gates the synaptic release of many neurotransmitters in the brain (Katona & Freund, 2012; Ohno-Shosaku & Kano, 2014). Cannabinoid type 1 (CB_1) receptors are abundantly expressed across glutamatergic, GABAergic, monoaminergic and neuropeptidergic neurons throughout the brain (Katona & Freund, 2012; Ohno-Shosaku & Kano, 2014). Activation of this receptor suppresses neurotransmitter release and contributes to multiple forms of synaptic plasticity that appear to be important for learning, adaptation, and other physiological processes including pain perception and feeding (Mechoulam & Parker, 2013; Melis et al., 2014). *N*-rachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) represent the two most characterized eCB molecules to date and are believed to be primarily formed in post-synaptic cells where they act in a retrograde fashion to modulate neurotransmitter release in response to a variety of intracellular signals, including increased calcium and activation of metabotropic receptors (Katona & Freund, 2012; Ohno-Shosaku & Kano, 2014). With respect to the regulation of stress, the eCB system is widely distributed throughout cortico-limbic circuits such as the prefrontal cortex (PFC), amygdala and hippocampus (Herkenham et al., 1991), that are important for the processing of emotionally salient information and the generation of neurobehavioral responses to stress (Davidson & McEwen, 2012).

In general, eCB signaling buffers against the effects of stress as disruption of this system results in exaggerated neurobehavioral and hormonal responses to stress, impairs appropriate termination of stress responses, compromises adaptation to stress and promotes structural changes in the brain associated with mood and anxiety disorders (Hill & Patel, 2013; Hillard, 2014; Morena et al., 2015). More so, there is a rapidly increasing body of clinical literature indicating that alterations in eCB signaling are associated with the development of psychiatric illnesses, such as depression and PTSD (see Hill et al., 2018; Hill & Patel, 2013; Hillard et al., 2012). Building on these findings, it has been well established that exposure to chronic stress results in a weathering of eCB signaling that compromises the stress-inhibitory role this system normally exerts, which in turn may contribute to the development of the pathological effects of chronic stress, also often referred to as allostatic load (Gorzalka et al., 2008; Hillard, 2014; Morena et al., 2016).

It is known that the eCB system undergoes dramatic reorganization during development, playing a prominent role in axon guidance and circuit formation in the perinatal period (Alpár et al., 2014; Berghuis et al., 2007; Mulder et al., 2008), followed by a progressive increase in the ability of eCBs to regulate synaptic transmission through early development (Liang et al., 2014; Zhu & Lovinger, 2010). The transition of adolescence to adulthood is also associated with alterations in the ability of eCB signaling to regulate excitatory and inhibitory transmission in regions such as the PFC and hippocampus (Heng et al., 2011; Kang-Park et al., 2007). Interestingly, several reports have indicated that the eCB system is responsive to stress exposure in early life (D'Asti et al., 2010; Llorente et al., 2008; Marco et al., 2013), including potential long-lasting effects that continue into adulthood (Atsak et al., 2018; Llorente-Berzal et al., 2013; López-Gallardo et al., 2012; Naudon et al., 2013). However, no studies to date have examined how the normal developmental trajectory of eCB signaling is influenced by exposure to stress during the early postnatal period. Given the importance of eCB signaling in the regulation of stress responses (Hill & Tasker, 2012; Morena et al., 2016), and the enhanced vulnerability to stress-related conditions that emerges in individuals following early life stress exposure, the aim of the current study was to characterize the immediate and sustained effects of neonatal stress exposure on the developmental trajectory of the eCB system.

Methods

Animals

Timed-pregnant Sprague-Dawley rats obtained from Charles River Laboratories (Kingston, NY) arrived at our animal facility on gestational day 13. The dams were singly housed. Two days after delivery (postnatal day 2; PND2), litters were culled to 12 pups with equal number of males and females where possible. Litters were randomly assigned to a control (CONT) or maternal separation (MS) group. Animals were maintained on a 12h light-dark schedule (lights on from 0600h to 1800h) and the ambient temperature was maintained at 21 ± 2°C. Food and water were available *ad libitum.* All protocols were approved by the Institutional Animal Care and Use Committee of Rockefeller University.

Maternal Separation

MS dams were removed from the home cage and their litters were transported in the home cage to a separate room and placed in a clean cage inside of a temperature-controlled isolette for a period of three hours a day on PND2-PND12. The temperature of the isolette was set at 32°C from PND2-PND6 and at 30°C from PND7-PND12. Additionally, pups were permitted to huddle with their littermates during the separation period. Previous studies demonstrate that under similar conditions, the core temperature of pups is maintained at 36-37°C (Jans & Woodside, 1990). Dams remained in the home cage undisturbed during the separation period. At the end of the three-hour separation period, litters were returned to the housing room and their respective dams were returned to the home cage. Non-maternally separated litters (CON) were left undisturbed with their dams from PND2-PND12. Twice weekly cage cleaning commenced on PND13 for all litters. All litters were weaned on PND20 and housed in same-sex groups of 2-3 rats per cage.

Tissue Harvest

All biochemical analyses were completed using male pups and only 2 pups per litter were used for any assay to prevent litter specific effects. Animals (n = 7-8 / treatment condition) were sacrificed by decapitation at the following developmental time points to assess eCB function: PND2 -immediately after the 1st MS session; PND12- immediately after the final MS session; PND14- juvenile (basal juvenile state, 2 days after the final MS session); PND40 adolescent basal state and PND70 adult basal state. All tissues from all treatment conditions at all age points were collected during the first 3 hours of the light cycle. Tissue was harvested and analyzed from cortico-limbic brain regions known to modulate the stress response: 1. the prefrontal cortex (PFC)- composed of medial prefrontal cortex and anterior cingulate; 2. the hippocampus - containing all subregions of the hippocampus, as well as both dorsal and ventral regions; and 3. the amygdala- composed of central, basolateral, and medial nuclei. The regions were anatomically identified as described previously (Hill et al., 2010). The PFC, hippocampus, and amygdala were dissected out on ice, immediately frozen in liquid nitrogen, and stored at −80°C degrees until analysis. Samples from all time points were used for analysis of eCB content, while only PND40 and PND70 (n = 4 / treatment condition) samples were used for CB_1 receptor binding density assays due to limitations from protein quantity and tissue size that are required for accurate assessment of CB_1 receptor binding site densities using this assay.

Analysis of Endocannabinoid Ligands

For analysis of eCB content, brain regions were subjected to a lipid extraction process as described previously (Patel et al., 2003). The contents of the two primary eCBs, AEA and 2-AG, within lipid extracts were determined using isotope-dilution liquid chromatography–mass spectrometry as described previously (Patel et al., 2005).

CB_1 Receptor Binding Assay

CB_1 receptor binding assays were performed using a Multiscreen Filtration System with Durapore 1.2-μM filters (Millipore, Bedford, MA) as described previously (Hillard et al., 1995). Incubations (total volume = 0.2 mL) were carried out using TME buffer containing 1

mg/mL bovine serum albumin (TME/BSA). Membranes (10 μg protein per incubate) were added to the wells containing 0.25, 0.5, 1.0, or 2.5 nM ^{3}H-CP 55,940. Ten μM Δ^{9}-tetrahydrocannabinol was used to determine non-specific binding. K_D and Bmax values were determined by nonlinear curve fitting to the single site binding equation using GraphPad Prism (San Diego, CA, USA).

Data Analysis

The effects of developmental age and exposure to MS on eCB ligand levels (AEA and 2-AG) and CB_1 receptor binding site densities were analyzed using a 2 factor analysis of variance, with age and stress exposure acting as fixed factors. Post hoc analysis was performed using a Tukeys test. Significance was established against an alpha level equal to 0.05. For all eCB measures an n = 8, and for CB_1 receptor binding an n = 4, was used per treatment condition at each age epoch.

Results

Developmental Trajectory of Endocannabinoid Content in the Prefrontal Cortex and its Modulation by Early Life Stress

Within the PFC, there was no interaction between age and MS on AEA content [$F(4, 68) = 0.11$, $p > 0.05$; Fig. 1], nor a main effect of MS [$F(1, 68) = 0.01$, $p > 0.05$], but there was a main effect of age [$F(4, 68) = 61.99$, $p < 0.001$], such that AEA levels at P40 and P70 were higher than at P2, P12 and P14 ($p < 0.001$ for all comparisons). With respect to 2-AG, there was no interaction between age and MS [$F(4, 69) = 1.76$, $p > 0.05$; Fig. 1], nor an effect of MS [$F(1, 68) = 0.06$, $p > 0.05$], but there was a main effect of age [$F(4, 68) = 56.90$, $p < 0.001$]. Unlike AEA, however, this was not a linear change, as 2-AG tissue levels in the PFC were significantly higher at P12 and P14, relative to P2, P40 and P70 ($p < 0.01$ for all comparisons).

Developmental Trajectory of Endocannabinoid Content in the Amygdala and its Modulation by Early Life Stress

Within the amygdala, there was a significant interaction between age and MS exposure [$F(4, 66) = 4.39$, $p < 0.01$; Fig. 2]; post-hoc analysis revealed both age and MS dependent changes in AEA content within the amygdala. With respect to age, AEA levels in the amygdala at P2 were significantly lower than at P12 ($p < 0.01$), P14 ($p < 0.01$), P40 ($p < 0.001$) and P70 ($p < 0.01$). Similarly, AEA levels in the amygdala at P12 and P14, while not different from each other ($p > 0.05$), were significantly lower than at P40 ($P < 0.001$) and P70 ($p < 0.001$). With respect to stress, however, there was no difference in AEA levels between control rats and those exposed to MS at P2 ($p > 0.05$), or at adolescence at P40 ($p > 0.05$) or adulthood at P70 ($p > 0.05$). However, immediately following the final session of MS stress (P12), and, 2 days following the final bout of MS (P14), AEA content within the amygdala in rats exposed to early life stress were significantly reduced compared to control rats (P12: $p < 0.01$; P14: $p < 0.05$).

There was a significant interaction between age and MS on amygdalar 2-AG content [$F(4, 67) = 3.25$, $p < 0.02$; Fig. 2]. Specifically, as was seen in the PFC, there was an age effect on

2-AG content, regardless of MS exposure, such that 2-AG contents in the amygdala at P12 and P14 were significantly higher than at P2, P40 and P70 (all comparisons $p < 0.01$). With respect to MS, it was the P12 and P14 ages when the impact of MS was apparent. Specifically, at P2, P40 and P70, there were no differences in 2-AG content in rats that had been exposed to early like stress versus control rats, but at both P12 ($p < 0.01$) and P14 ($p < 0.05$), animals that had been exposed to MS exhibited higher levels of 2-AG than control rats.

Developmental Trajectory of Endocannabinoid Content in the Hippocampus and its Modulation by Early Life Stress

Within the hippocampus, there was no significant interaction between exposure to MS and age on AEA content [$F (4, 67) = 2.10$, $p > 0.05$; Fig. 3]. There was a main effect of MS [$F (1, 67) = 19.91$, $p < 0.001$], such that exposure to early life stress caused a general reduction in AEA content in the hippocampus at all ages. There was also a main effect of age [$F (4, 67) = 79.22$, $p < 0.001$], similar to what was seen in other structures, such that AEA content in the hippocampus was higher at P40 and P70 relative to all younger ages ($p < 0.05$ for all comparisons). There was a significant interaction between MS exposure and age on 2-AG content in the hippocampus [$F (4, 68) = 5.20$, $p < 0.01$; Fig. 3]. Unlike the PFC and amygdala, 2-AG changed in a more linear manner across ages; 2-AG content was significantly higher at P40 and P70 relative to all younger ages ($p < 0.05$ for all comparisons). However, when post-hoc analysis examined the impact of MS exposure, the effects were very age dependent. Specifically, at P2, immediately after the first exposure to MS stress, there was a significant reduction in 2-AG content in the hippocampus in the stressed rats relative to the control rats ($p < 0.05$). Immediately after the final MS exposure (P12), 2-AG levels in the hippocampus were significantly higher in the stressed animals relative to the control animals ($p < 0.05$). At P14, there were no differences in 2-AG content between the two groups in the hippocampus. By P40 and continuing to P70, 2-AG levels within the hippocampus were significantly lower in the animals that had experienced MS, relative to the control animals ($p < 0.05$ and $p<0.01$, respectively).

The impact of early life stress on CB_1 receptor binding site densities in adolescence and adulthood.

Within the PFC, there was no interaction between MS exposure and age on the Bmax of the CB_1 [$F (1, 12) = 0.78$, $p > 0.05$; Fig. 4], nor an effect of age [$F (1, 12) = 0.16$, $p > 0.05$], but there was a significant main effect of MS exposure [$F (1, 12) = 6.46$, $p < 0.03$], such that exposure to MS was related to reductions in CB_1 receptor binding site density in the PFC, at both ages, relative to control rats. A similar effect was seen with CB_1 receptor binding affinity, where there was no interaction between MS and age [$F (1, 12) = 1.50$, $p > 0.05$], nor a main effect of age [$F (1, 12) = 0.44$], but there was a main effect of MS exposure [$F (1, 12) = 5.26$, $p < 0.05$], such that regardless of age, exposure to MS reduced the Km for the CB_1 receptor (P40 CON: 0.63 nM +/− 0.10; P40 MS: 0.26 nM +/− 0.04; P70: CON 0.43 nM +/− 0.1; P70 MS: 0.32 nM +/− 0.12).

A similar pattern emerged within the amygdala, such that there was no interaction between age and MS exposure on the Bmax of the CB_1 receptor [$F (1, 12) = 0.53$, $p > 0.05$; Fig. 4],

or a main effect of age [F (1, 12) = 0.47, p > 0.05]; but there was a significant main effect of MS exposure [F (1, 12) = 12.65, p < 0.005], such that regardless of age, exposure to MS reduced the Bmax of the CB1 receptor within the amygdala. However, there was no significant interaction [F (1, 12) = 0.01, p > 0.05], no main effect of age [f (1, 12) = 0.13, p > 0.05] nor a significant main effect of MS exposure [F (1, 12) = 3.73, p > 0.05] on the binding affinity of the CB_1 receptor in the amygdala (P40 CON: 0.47 nM +/− 0.15; P40 MS: 0.20 nM +/− 0.12; P70 CON: 0.51 nM +/− 0.21; P70 MS: 0.26 nM +/− 0.04).

Analysis of the binding parameters of the CB_1 receptor within the hippocampus revealed a main effect between age and MS exposure [F (1, 12) = 4.97, p < 0.05; Fig 4], with post hoc analysis demonstrating effects of both age and MS. Specifically, there was an increase in the Bmax of the CB_1 receptor between P40 and P70 (p < 0.05) in control animals. While there was no difference between the Bmax of the CB_1 receptor at P40 in control rats versus those that were exposed to MS (p > 0.05), at P70, rats exposed to MS exhibited a significant reduction in CB_1 receptor binding sites relative to control rats (p < 0.05). With respect to the binding affinity of the CB_1 receptor, there was no interaction between age and MS exposure [F (1, 12) = 3.16, p > 0.05], nor an effect of age [F (1, 12) = 0.70, p > 0.05], there was a main effect of stress [F (1, 12) = 9.25, p < 0.02], such that, regardless of age, there was a reduction in the binding affinity of the CB_1 receptor in rats that had been exposed to MS (P40 CON: 0.62 nM +/− 0.10; P40 MS: 0.52 nM +/− 0.06; P70 CON: 0.84 nM +/− 0.05; P70 MS: 0.44 nM +/− 0.10).

Discussion

This study demonstrated that exposure of neonatal rats to MS stress from PND2-12 resulted in both immediate and sustained effects on the corticolimbic eCB system. Generally, dynamic stress-induced changes in neonatal eCB levels are consistent with changes documented in adult rats (Morena et al., 2016), suggesting that the eCB system is responsive to stress, even during the early life period. This is consistent with the limited data available from previous reports indicating that eCB signaling is modulated by stress during early development (D'Asti et al., 2010; Llorente et al., 2008; Marco et al., 2013). More so, it is likely that these eCB changes are functionally relevant to the effects of stress since previous work has demonstrated that eCB signaling can regulate both behavioral and neuroendocrine responses to stress at this early developmental window (Buwembo et al., 2013; D'Asti et al., 2010; Fride et al., 2005). It should be noted that these changes were only seen after the final stress exposure, however, due to low tissue concentrations of AEA at PND2, and the possibility that rapid changes could have occurred which may have habituated by the conclusion of the 3h separation period, we cannot rule out that the first exposure to separation stress also had an impact on eCB signaling in the neonates.

Impact of Early Life Stress on the Endocannabinoid System within the PFC

Tracking changes in the eCB system across development, following exposure to MS, revealed that stress exposure in early life modulates the normative ontogeny of the eCB system in a region-specific manner. Quite surprisingly, the PFC appeared entirely resistant to any impact of early life stress on eCB content at any of the time points measured, but did

exhibit a persistent downregulation of CB_1 receptors in adolescence and adulthood in animals exposed to MS. This reduction in prefrontal CB_1 receptor binding sites after developmental exposure to stress mirrors data from our group that exposure to stress during adolescence also resulted in a sustained downregulation of prefrontal cortical CB_1 receptors in adulthood (Lee & Hill, 2013). The functional relevance of these changes is not yet known, however, given the importance of CB_1 receptor signaling within the PFC to regulate dendritic plasticity (Hill et al., 2011a), termination of the stress response (Hill et al., 2011b) and emotional behavior (McLaughlin et al., 2014; McLaughlin et al., 2012; Rubino et al., 2008), all of which are influenced by developmental exposure to stress, we hypothesize that these changes are functionally related.

Impact of Early Life Stress on the Endocannabinoid System within the Amygdala

Repeated exposure to MS stress in neonates produced similar changes in eCB content as to what is seen in adults exposed to chronic stress (Morena et al., 2016), whereby repeated exposure to MS stress resulted in a reduction in AEA content throughout the juvenile period (PND12-14), coupled to an increase in 2-AG content at the same time points. This suggests that the effect of stress on eCB signaling within the amygdala is preserved in early life, which is interesting given the importance of amygdalar eCB signaling to various aspects of the stress response, including activation of the HPA axis and the generation of an anxious state. Similar to the PFC, CB_1 receptor binding site densities within the amygdala were downregulated both at adolescence and adulthood in animals exposed to MS, despite normalization of eCB content. While not examined in this study, an impairment in CB_1 receptor signaling within the amygdala in adulthood would likely render an organism more sensitive to the adverse effects of stress. Future work will have to examine a “double hit” approach to establish if the downregulation of amygdalar CB_1 receptors contributes to the altered stress sensitivity animals exposed to early stress exhibit in adulthood.

Impact of Early Life Stress on the Endocannabinoid System within the Hippocampus

Within the hippocampus, there was a general effect of MS stress such that AEA levels were reduced at every single developmental time point following MS stress, indicating an exquisite sensitivity of AEA signaling in the hippocampus to early life adversity. 2-AG content, on the other hand, was reduced in response to the first bout of neonatal stress, but was then transiently elevated after the final exposure to MS stress, only to return to baseline levels by PND14. By adolescence, and into adulthood, however, 2-AG content exhibited a prominent reduction in tissue levels in animals exposed to MS indicating that early life stress resulted in a profound, and sustained, reduction in both eCB ligands within the hippocampus at later developmental windows. Unlike what was seen in the PFC and amygdala, however, there was no impact of MS stress on CB_1 receptor binding site densities at adolescence. By adulthood, however, when control animals exhibited a significant elevation in CB_1 receptor density relative to adolescence, rats exposed to early life stress exhibited no such developmental change, and accordingly, there was a significant downregulation of CB_1 receptors in the hippocampus of these animals. Together, these data indicate that the most profound effects of early life stress on the eCB system appeared to be within the hippocampus where every aspect of the system (the primary receptor and both ligands) exhibited a significant downregulation, relative to animals reared under standard conditions.

Previous work has demonstrated that the hippocampus is particularly sensitive to the effects of early life stress (Andersen & Teicher, 2008; Brunson et al., 2003), such that basal changes in the hippocampus of adult rodents exposed to early life stress appear to mirror dynamic changes that are induced by exposure to chronic stress as adults, such as reductions in neurogenesis, neuronal complexity, dendritic arborization and spine densities (Andersen & Teicher, 2004; Brunson et al., 2005; Eiland & McEwen, 2012; Ivy et al., 2010; Karten et al., 2005; Leslie et al., 2011; Wang et al., 2011, 2013). These changes in hippocampal plasticity and architecture following early life stress are met by alterations in hippocampalmediated cognitive function (Brunson et al., 2005, 2003; Wang et al., 2011), including findings from our group using this model where we have demonstrated persistent impairments in object recognition memory in adults (Eiland & McEwen, 2012). Consistent with these findings, our data demonstrate that exposure to early life stress causes a robust impairment in the adult hippocampal eCB system (downregulation of CB_1 receptors as well as reduced AEA and 2-AG contents), which parallels data indicating that exposure to chronic stress in adulthood causes an impairment in eCB signaling within the hippocampus (Hill et al., 2005, 2008; Hu et al., 2011; Lee & Hill, 2013; Reich et al., 2009; Wang et al., 2014). One interpretation of these data is that the adult hippocampus of rodents exposed to early life stress appears to exist in a persistent state of allostatic load. As eCB signaling is a mediator of both synaptic and structural plasticity in the hippocampus (Chevaleyre & Castillo, 2004; Monory et al., 2015), these findings could suggest that exposure to early life stress compromises plasticity-related mechanisms within the hippocampus rendering this structure highly inflexible to experiential change in adulthood, which is consistent with a state of allostatic load (McEwen, 2007). The particular sensitivity of the hippocampus to these effects could relate to the timing of stress exposure. Specifically, several studies have demonstrated that the hippocampus is undergoing significant development during this early life window, in both animals and humans, and is accordingly, highly susceptible to the long-term effects of stress exposure (Andersen & Teicher, 2008; Loi et al., 2014; McClelland et al., 2011; Qiu et al., 2013).

It is interesting to note, that for measures of CB_1 receptor densities, almost all of the reductions in CB_1 receptor binding site density were coupled to an enhancement of the binding affinity for ligand binding to the CB_1 receptor. The relevance of these differential effects on CB_1 receptor density and affinity is not clear at this time, but the increased affinity could be a compensatory mechanism attempting to counter the reduced level of receptor. Future studies employing electrophysiology will be required to determine the impact of early life stress on CB_1 receptor functionality and the neuronal populations on which this occurs. Regardless, these findings are generally consistent with data generated using a similar model of maternal deprivation where early life stress produced reductions in CB_1 receptor expression (using immunohistochemistry) in the adult hippocampus (López-Gallardo et al., 2012) and reductions in CB_1 receptor expression in the adolescent frontal cortex (Romano-López et al., 2012).

Normative Developmental Trajectory of the Endocannabinoid System

Examination of eCB content across multiple developmental epochs allowed us to determine a general trajectory for the normative ontogeny of this system (also see Lee and Gorzalka,

2012 for review of previous work on development of the eCB system, particularly during discrete periods of adolescence which was not assessed to the same degree herein). Both the amygdala and PFC appeared to exhibit a comparable trajectory whereby AEA levels were almost undetectable at PND2, increase slightly at PND12-14 and then dramatically increase by PND40 and 70. 2-AG, on the other hand, was comparable to adult levels at PND2, but then exhibited a dramatic elevation in tissue levels on PND12-14, declining to adult levels at PND40 and 70. The comparable levels of 2-AG in neonates and adults, but progressive increase in AEA levels is consistent with a previous report (Berrendero et al., 1999), however, the current study extends these investigations into the juvenile period where a robust increase in 2-AG content is observed. The functional nature of these evolving eCB levels remains elusive, but it is interesting to note that the developmental window in which 2-AG levels appear to be higher in corticoamygdala circuits is during a period of significant development in this circuit and approximately corresponds to the period when the amygdala develops the ability to assign negative valence to stimuli and recognize aversive cues (Barr et al., 2009; Moriceau & Sullivan, 2006; Sullivan & Holman, 2010). Given that 2-AG signaling during development has been identified to influence axon guidance and circuit formation (Keimpema et al., 2010, 2013; Oudin et al., 2011; Wu et al., 2010), the possibility exists that this signal could be involved in the development of the frontocortical-amygdala circuit that is essential for the top down control of emotional behavior and valence detection. Stress exposure early in life has been shown to accelerate the maturation and ability of the amygdala to respond to aversive cues (Gee et al., 2013; Moriceau et al., 2009), and in the current study, stress exposure significantly increased 2-AG content within the amygdala during this window, suggesting that future work should examine whether there is any role for 2-AG in the maturation of the amygdala, its connections with the frontal cortex and whether stress-induced changes in eCB signaling are relevant to stress exposure's known ability to modulate maturational processes within this circuit (Gee et al., 2013; Malter Cohen et al., 2013). Of note, the ontogeny of hippocampal eCB signaling did not parallel the developmental trajectories of eCB signaling in the amygdala and PFC in that hippocampal AEA and 2-AG levels appeared to increase progressively in a linear manner from birth into adulthood. While the relevance of the ontogenetic dissociation between these structures is currently unknown, it does suggest that there are differential trajectories of eCB signaling across the brain and that future work should examine this profile in more depth to understand both the mechanisms and relevance of these differences.

Limitations and Considerations

A limitation of this study was that all of the analysis was only performed in male, and not female rats. To date, there is almost no information available regarding how stress, even in adulthood, modulates eCB content in females relative to males. Ongoing research in our group is beginning to investigate sex-specific changes in eCB content and CB_1 receptors in response to stress. Once a more comprehensive understanding of the sex-specific nature by which stress modulates eCB signaling is established, the developmental effects of stress will also have to be investigated. This is particularly relevant given that sex differences in eCB signaling have been widely established, both in a developmental context as well as in adulthood (Huang & Woolley, 2012; Krebs-Kraft et al., 2010; Tabatadze et al., 2015). Similarly, another issue of consideration in the interpretation of these data is that all of the

pregnant dams were exposed to shipping during gestational day 10-12 of pregnancy. The stress of shipment during this period of gestation has been found to impact neural adaptations to additional stimuli (Ogawa et al., 2007; Moriyama et al., 2013), and thus could have an impact on the generalization of the data generated herein.

Conclusions

Collectively this study highlights a previously undetermined non-linear and region-specific developmental trajectory of the eCB system within corticolimbic structures that is influenced by exposure to stress during the postnatal period. The hippocampus appears to be particularly sensitive to these sustained effects into adulthood, which could be due to its stage of development during the stress exposure. This is consistent with other studies identifying the PND2-12 window as being particularly sensitive to manipulations, such as elevated 5-HT signaling (Rebello et al., 2014), which produce sustained alterations in neurodevelopment that continue into adulthood. These data also indicate that the eCB system in adulthood is sensitive to programming by early life experience. Given the important stress-inhibitory role of eCB signaling, the ability of early life stress to compromise this system could represent one mechanism by which early life stress increases vulnerability to stress-related psychiatric illnesses in adulthood. In this light, it is interesting to note that gene variants in the CB_1 receptor, and enzymes which metabolizes AEA and 2-AG, have been shown to influence the effects of early life stress on the development of anhedonia, stress sensitivity and depression in adulthood (Agrawal et al., 2012; Carey et al., 2015; Lazary et al., 2016).

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Highlights

- Maternal separation stress modulates endocannabinoid levels in neonates
- Endocannabinoid signaling in the adult hippocampus is impaired by early life stress
- Early life stress does not impact cortical and amygdala endocannabinoid levels in adulthood

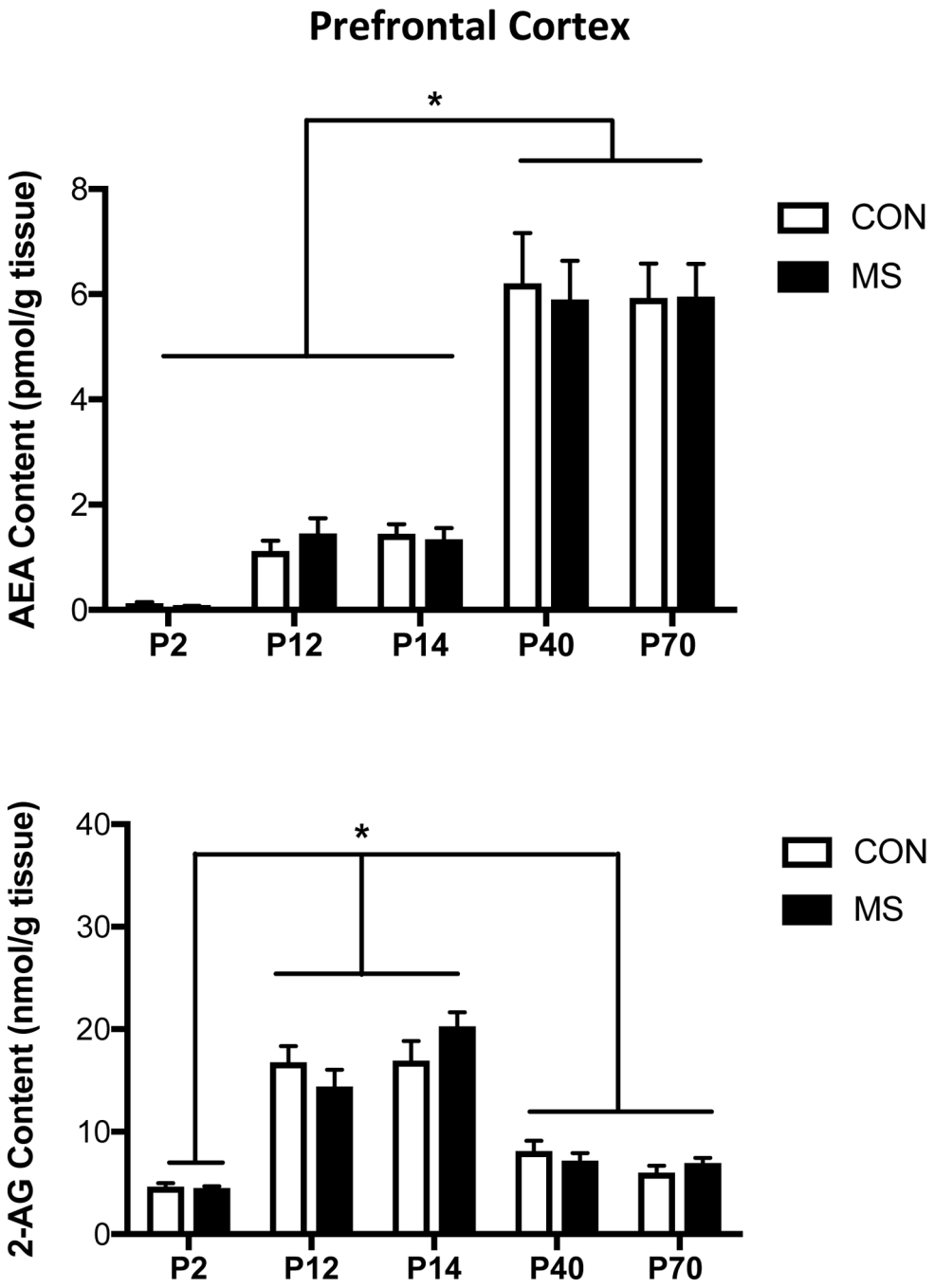

Figure 1. The Effect of Maternal Separation Stress on the Developmental Trajectory of the Endocannabinoid Contents in the Prefrontal Cortex

Tissue content of the endocannabinoid anandamide (AEA; **upper panel**) is found to elevate in the prefrontal cortex (PFC) at postnatal day (PND) 40 and 70 relative to PND 2, 12 and 14. This developmental change was uninfluenced by exposure to 3 hours of maternal separation (MS) stress per day from PND 2-11. 2-arachidonoylglycerol (2-AG; **lower panel**), the other primary endocannabinoid molecule, exhibited a different developmental trajectory where it was found to be significantly higher at PND 12 and 14 relative to both PND 2 as well as PND 40 and 70. Again, there was no influence of MS stress on PFC levels

of 2-AG at any age, relative to control (CON) animals. Data are presented as means +/− SEM. * denotes significant differences ($p < .05$) between identified age windows. All n = 7-8 / treatment condition.

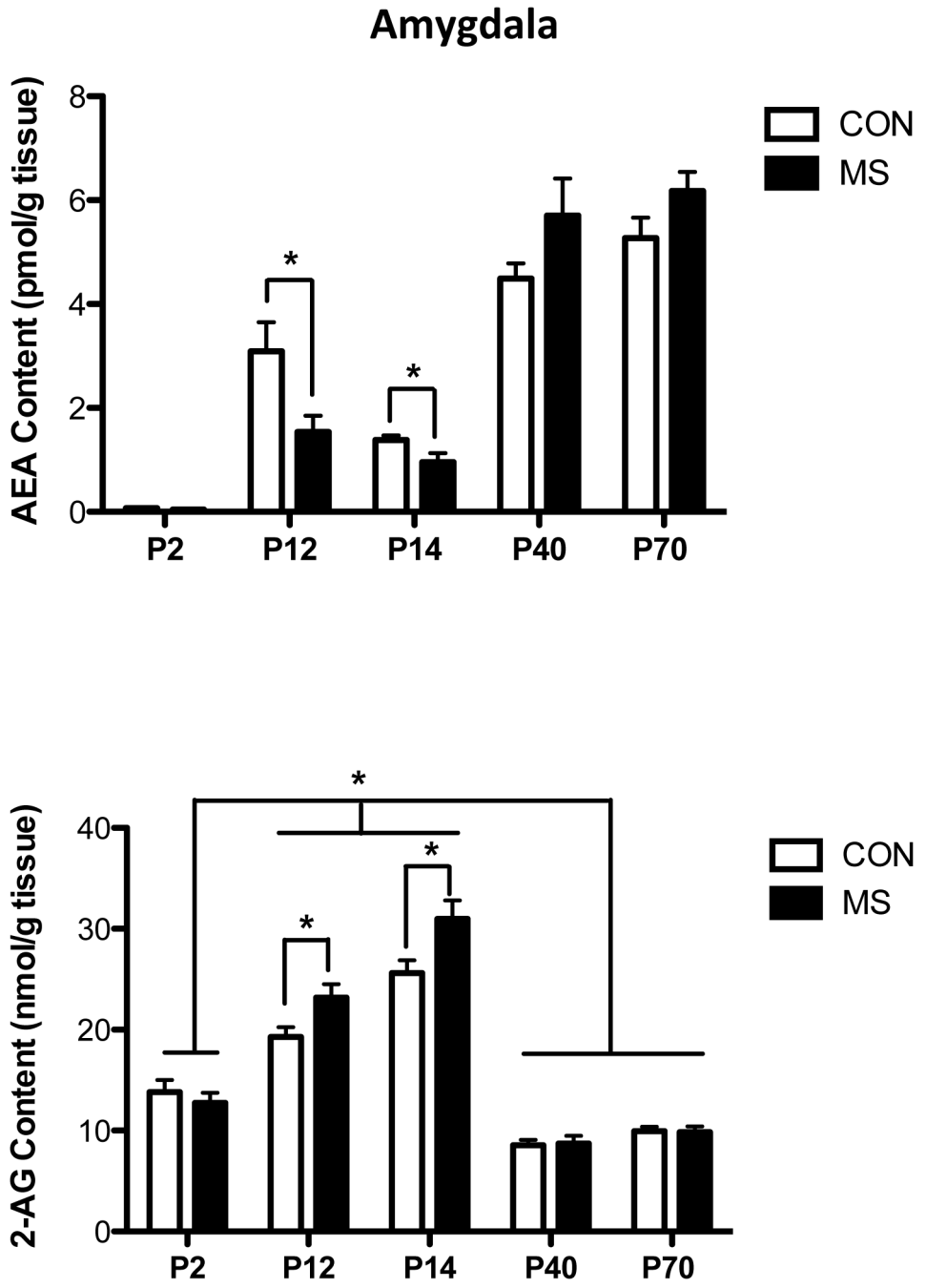

Figure 2. The Effect of Maternal Separation Stress on the Developmental Trajectory of the Endocannabinoid Contents in the Amygdala

Tissue content of the endocannabinoid anandamide (AEA; **upper panel**) was found to elevate in the amygdala in an age-dependent manner, with AEA content being higher at PND 12 and 14, relative to PND2, while AEA content at P40 and P70 was higher than at the earlier time points. Exposure to 3 hours of maternal separation (MS) stress per day from PND 2-11 resulted in significant reductions in AEA content in the amygdala both at PND 12 and PND 14, relative to control (CON) animals. These effects had normalized by PND40 and 70. 2-arachidonoylglycerol (2-AG; **lower panel**), the other primary endocannabinoid

molecule, exhibited a different developmental trajectory where it was found to be significantly higher at PND 12 and 14 relative to both PND 2 as well as PND 40 and 70. Similar to AEA, but in the opposite direction, exposure to MS stress from PND2-11 resulted in an elevation in 2-AG content in the amygdala at both PND12 and PND14. Data are presented as means +/− SEM. * denotes significant differences ($p < .05$) between identified age windows or between identified CON and MS groups. All n = 7-8 / treatment condition.

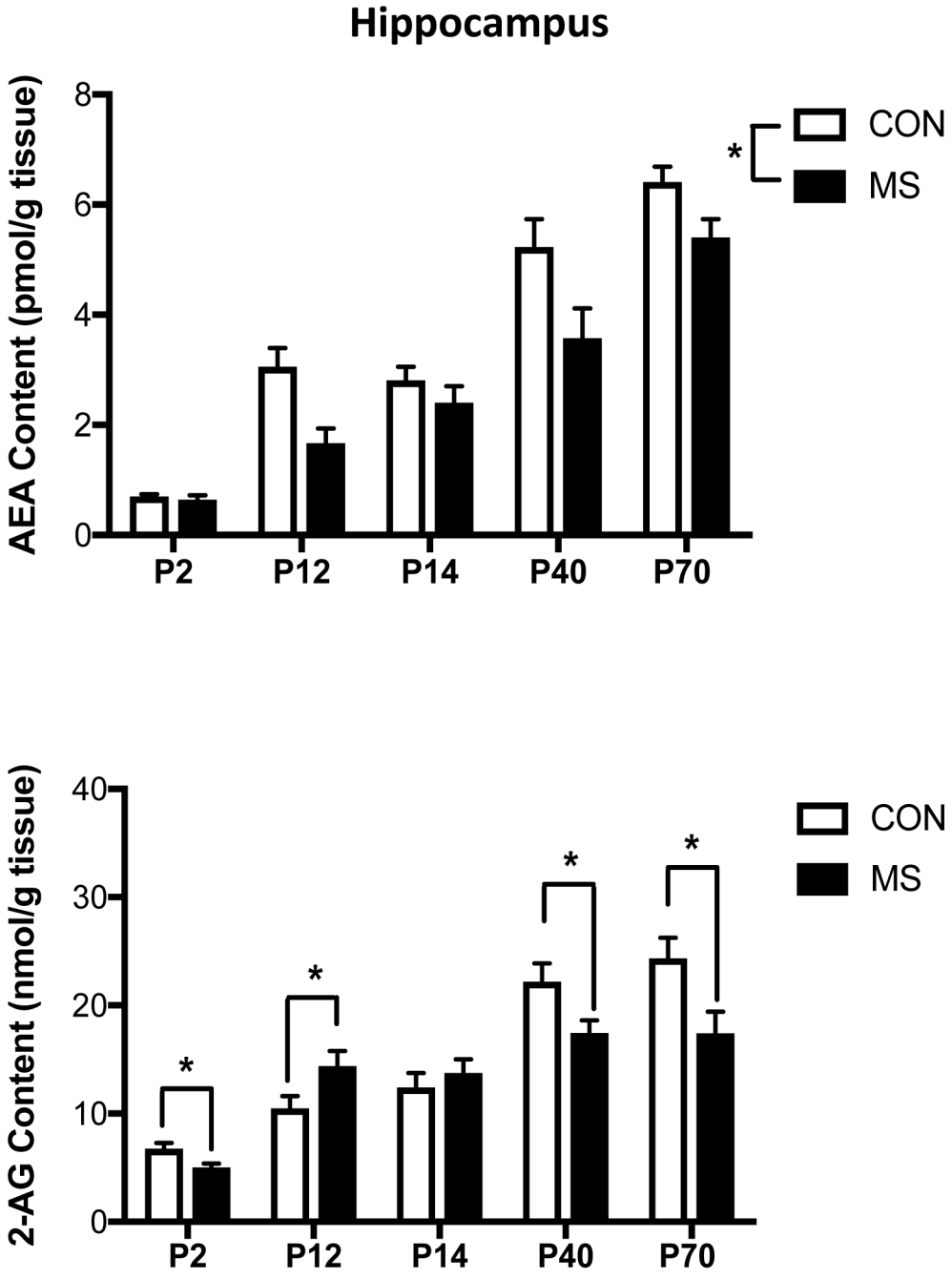

Figure 3. The Effect of Maternal Separation Stress on the Developmental Trajectory of the Endocannabinoid Contents in the Hippocampus

Tissue content of the endocannabinoid anandamide (AEA; **upper panel**) was found to elevate in the hippocampus in an age-dependent manner, with AEA content being higher at postnatal day (PND) 40 and 70 relative to PND 2, 12 and 14. Exposure to 3 hours of maternal separation (MS) stress per day from PND 2-11 resulted in a main effect of reduced in AEA content in the hippocampus across all age points. 2-arachidonoylglycerol (2-AG; **lower panel**), the other primary endocannabinoid molecule, was also found to be higher at postnatal day (PND) 40 and 70 relative to PND 2, 12 and 14 in the hippocampus. Exposure

to MS stress had age specific effects on 2-AG content in the hippocampus, with 2-AG levels significantly reducing on PND2 after the first exposure to MS, but then elevating on PND12 after the last MS stress, relative to control (CON) animals. These effects had normalized by PND 14, but then again at both PND40 and PND70 tissue levels of 2-AG in the hippocampus were found to be significantly reduced in rats which had been exposed to MS stress relative to CON animals. Data are presented as means +/− SEM. * denotes significant differences ($p < .05$) between identified CON and MS groups. All n = 7-8 / treatment condition.

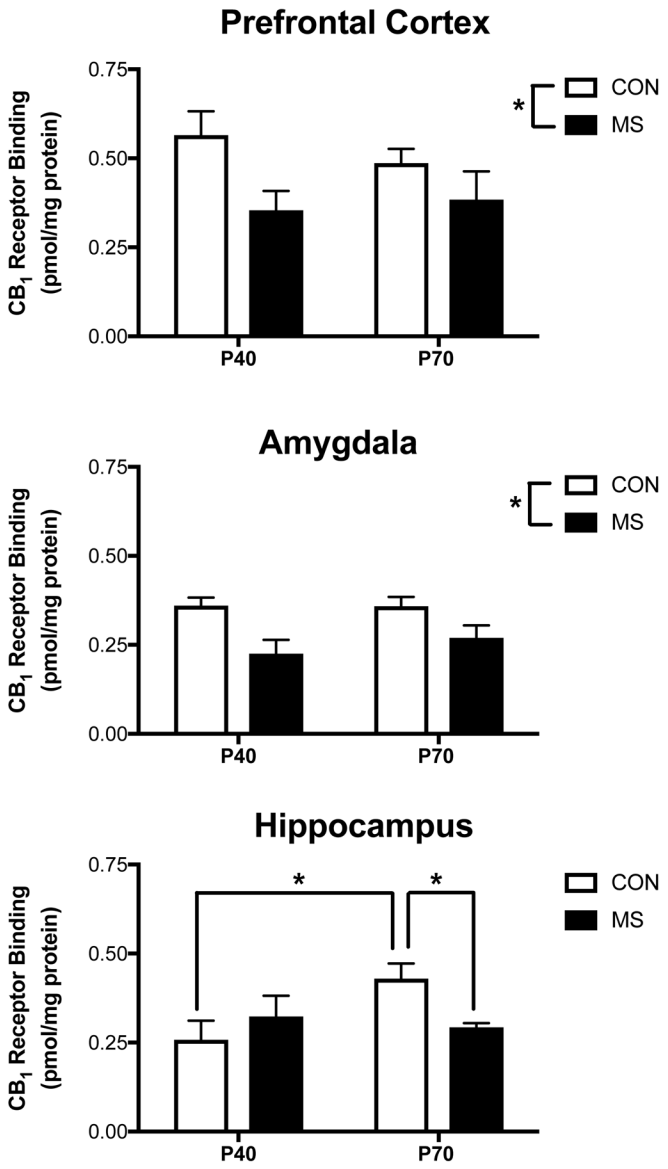

Figure 4. Maternal Separation Impacts CB_1 Receptor Densities in the Adolescent and Adult Brain

The maximal binding site density (B_{max}) of the cannabinoid CB1 receptor, as determined by specific binding of the ^{3}H-CP55,940, was examined in the prefrontal cortex, amygdala and hippocampus of the adolescent brain at PND40 and the adult brain at PND70. Within the prefrontal cortex (**upper panel**) and amygdala (**middle panel**), maternal separation (MS) stress was found to result in a main effect of reduced CB1 receptor binding site densities at both PND40 and PND70. Within the hippocampus, there was an age dependent effect, such that CB1 receptor binding site densities were found to be higher at PND70 relative to PND40. Exposure to MS stress had no effect on CB_1 density within the hippocampus at PND40, but at PND70 animals exposed to MS stress had reduced CB_1 receptor binding site densities relative to control (CON) animals. Data are presented as means +/− SEM. *

denotes significant differences ($p < .05$) between CON and MS groups or identified age groups. All n = 4 / treatment condition.