Neuropharmacology 99 (2015) 89–97

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Disruption of peri-adolescent endocannabinoid signaling modulates adult neuroendocrine and behavioral responses to stress in male rats

Tiffany T.-Y. Lee [a], Matthew N. Hill [b], Cecilia J. Hillard [c], Boris B. Gorzalka [a, *]

[a] Dept. of Psychology, University of British Columbia, Vancouver, V6T 1Z4, Canada
[b] Hotchkiss Brain Institute, Dept. of Cell Biology and Anatomy, University of Calgary, Calgary, AB T2N 4N1, Canada
[c] Dept. of Pharmacology and Toxicology and Neuroscience Research Center, Medical College of Wisconsin, Milwaukee, WI 53226, USA

ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form
14 July 2015
Accepted 16 July 2015
Available online 17 July 2015

Keywords:
Adolescence
HPA axis
AM-251
CB1 receptor
Anandamide
2-AG
Elevated plus maze
Forced swim test
Stress reactivity

ABSTRACT

The endocannabinoid (eCB) system is known to regulate neural, endocrine and behavioral responses to stress in adults; however there is little knowledge regarding how this system governs the development and maturation of these responses. Previous work has reported dynamic and time-specific changes in CB_1 receptor expression, *N*-arachidonylethanolamine (AEA) content and fatty acid amide hydrolase (FAAH) activity within corticolimbic structures throughout the peri-adolescent period. To examine whether fluctuations in adolescent eCB activity contribute to the development of adult stress responsivity and emotionality, we treated male Sprague–Dawley rats daily with the CB_1R antagonist, AM-251 (5 mg/kg), or vehicle between post-natal days (PND) 35–45. Following this treatment, emotional behavior, HPA axis stress reactivity and habituation to repeated restraint stress, as well as corticolimbic eCB content were examined in adulthood (PND 75). Behaviorally, AM-251-treated males exhibited more active stress-coping behavior in the forced swim test, greater risk assessment behavior in the elevated plus maze and no significant differences in general motor activity. Peri-adolescent AM-251 treatment modified corticosterone habituation to repeated restraint exposure compared to vehicle. Peri-adolescent CB_1R antagonism induced moderate changes in adult corticolimbic eCB signaling, with a significant decrease in amygdalar AEA, an increase in hypothalamic AEA and an increase in prefrontal cortical CB_1R expression. Together, these data indicate that peri-adolescent endocannabinoid signaling contributes to the maturation of adult neurobehavioral responses to stress.

1. Introduction

Accumulating evidence suggests that the adolescent brain undergoes region-dependent maturation in key higher-order processing structures, such as the prefrontal cortex (PFC; e.g. Casey and Jones, 2010; Gogtay et al., 2004). However, one consequence of the highly plastic nature of these maturational neural processes is that developing neural circuits are vulnerable to perturbations, such as stress exposure and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Adriani and Laviola, 2004; Andersen, 2003). Preclinical studies suggest that adolescent stress exposure elicits detrimental long-term behavioral and neural alterations, depending on stressor type, duration and age of exposure. Indeed, peri-adolescent stress exposure is known to alter HPA axis stress responsivity (Ver Hoeve et al., 2013), decrease social interaction (McCormick et al., 2015), increase anxiety-like, depressive-like (McCormick et al., 2013) and aggressive behaviors (Márquez et al., 2013), facilitate amphetamine- and ethanol-stimulated locomotion, preference and self-administration (Burke and Miczek, 2014), alter reproductive behavior (McCormick et al., 2013) and impair working memory (Novick et al., 2013) during adulthood. Similarly, adolescent stress exposure leads to long-term neural changes including modifications to adult hippocampal electrophysiological and morphological profiles (Buwalda et al., 2005), altered amygdalar/orbitofrontal connectivity (Márquez et al., 2013), reduced hippocampal neurogenesis (Barha et al., 2011), and reduced prefrontal cortical synaptic density (Leussis et al., 2008).

The endocannabinoid (eCB) system, which interacts with the psychoactive constituents of cannabis, regulates a variety of processes in adulthood, including HPA axis stress responsivity (Hill and Tasker, 2012), emotional behavior (Kathuria et al., 2003; Marco and

* Corresponding author. Department of Psychology, University of British Columbia, 2136 West Mall, Vancouver, B.C. V6T1Z4, Canada.
E-mail address: bgorzalka@psych.ubc.ca (B.B. Gorzalka).

http://dx.doi.org/10.1016/j.neuropharm.2015.07.021
0028-3908/

Laviola, 2012; McLaughlin and Gobbi, 2011), reproductive behavior (Gorzalka et al., 2010) and drug dependence (Parolaro et al., 2007). The eCB system consists of two G-protein coupled receptors, CB_1 and CB_2. The CB_1 receptor (CB_1R) is widely expressed in the brain on neuron terminals (Herkenham et al., 1991), where it plays a regulatory role in synaptic function and plasticity (Castillo et al., 2012). CB_2 receptors are predominantly found in peripheral tissues, although reports have documented neuronal localization of CB_2 receptors in the central nervous system (Van Sickle et al., 2005). The eCB system also possesses two major endogenous ligands, *N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG), which are synthesized "on demand" and act as retrograde messengers to regulate the release of other neurotransmitters (Ahn et al., 2008). These actions contribute to both short and long term forms of synaptic plasticity (Mackie, 2006). Lastly, eCB signaling is highly regulated by metabolic enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase which hydrolyze AEA and 2-AG, respectively.

Moderate to high densities of CB_1Rs are found in adult corticolimbic structures regulating stress responsivity and emotional behavior (Herkenham et al., 1991) and evidence suggests that pharmacological antagonism and genetic deletion of CB_1Rs emulate a phenotype similar to that of chronic stress exposure, including heightened emotionality (Haller et al., 2002), hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Barna et al., 2004) and shorter, less complex pyramidal neurons in the medial PFC (Hill et al., 2011a; Lee et al., 2014). Moreover, CB_1R expression peaks in male rodents across several corticolimbic brain regions with the onset of adolescence (PND 30) and then gradually decreases to adult levels by PND 70 (Heng et al., 2011; Rodriguez de Fonseca et al., 1993). Simultaneously, AEA content and FAAH activity dynamically fluctuate throughout the adolescent period to adulthood (Ellgren et al., 2008; Lee et al., 2013; Rubino et al., 2015; Wenger et al., 2002). Thus, perturbations to these normative fluctuations in eCB signaling may compromise neurodevelopmental processes resulting in sustained changes in the adult brain (Rubino et al., 2015). The results of several studies indicate that peri-adolescent cannabinoid administration alters the developmental trajectory of corticolimbic structures, provoking profound, permanent, and often deleterious effects on cognition, emotionality and stress responsivity (Ceci et al., 2014; Lee and Gorzalka, 2012; Macrì et al., 2012; Rubino and Parolaro, 2008). Recently, these effects have been attributed to the possibility that excess exposure to cannabinoids during adolescence impairs ongoing eCB signaling through a desensitization of the system, and it is this disruption that mediates the adverse effects of cannabinoid exposure during adolescence (Rubino et al., 2015). However, the long-term consequences of adolescent CB_1R blockade in the development of adult HPA axis stress responsivity and emotional behavior remain unclear. To this end, we sought to unmask the contribution of normative adolescent CB_1R signaling in emotional behavior and HPA axis stress responsivity via pharmacological disruption of peri-adolescent eCB signaling.

2. Material and methods

2.1. Subjects

Male Sprague–Dawley rats (Charles River, QC, Canada) were received on post-natal day (PND) 21. Rats were pair housed in clear polyurethane cages (48 × 27 × 20 cm) filled with cedar bedding and paper towels for enrichment. A 12 h/12 h light/dark cycle (lights on at 9 am) was maintained and access to food (Purina rat Chow) and water was provided *ad libitum*. All efforts were made to minimize animal suffering and reduce the number of rats used, and protocols were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee at the University of British Columbia.

2.2. Experimental procedures

The CB_1R antagonist/inverse agonist, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM-251; Cayman Chemicals, Pittsburgh, PA), was dissolved in a vehicle of 10% DMSO, 10% Tween-80 and 80% 0.9% saline. A dose of 5 mg/kg AM-251 was chosen based on previous literature reporting that acute exposure at this dose produces some behavioral indices of stress exposure in adult rodents such as increased anxiety-like behavior (Litvin et al., 2013; Naderi et al., 2008; Sink et al., 2010). Thus, in the current study a moderate dose of 5 mg/kg AM-251 was used and intraperitoneally (IP) injected (or an equivalent volume of vehicle) in male rats from PND 35–45, the same cannabinoid administration period used by several studies conducted by the Parolaro laboratory (Rubino et al., 2015, 2009a, 2009b, 2008; Zamberletti et al., 2014). Rats were left undisturbed until PND 75, at which time they were randomly assigned to three different cohorts for analyses of: 1) emotional behavior, 2) HPA axis stress reactivity and 3) eCB content and expression throughout corticolimbic structures (all n = 10; see Fig. 1).

2.2.1. Emotional behavior assessment

Adult male rats were exposed to three behavioral tests (same sequence), always under dim lighting conditions, separated by 1 week each. In other words, rats in this cohort were exposed to the elevated plus maze on PND 75, the forced swim test on PNDs 82–83 and the open field test on PND 90. Experimenters blind to treatment condition scored all rat behavior by video.

2.2.1.1. Elevated plus maze. The elevated plus maze was employed in this study to assess the impact of peri-adolescent AM-251 treatment on adult anxiety-like behavior. It was constructed of painted black wood and consisted of 2 open arms (50 × 12.5 cm) and 2 enclosed arms (50 × 12.5 × 50 cm) that all extended from a common middle platform. The open, closed and middle platform were elevated 60 cm above the ground with 4 pedestals. Rats were placed in the middle platform and their behavior recorded for 5 min by an overhead camera approximately 2 m above the apparatus. A 10% acetic acid solution was used to clean the apparatus between rats. Number of entries into open and closed arms, time spent in open and closed arms, head dips and stretch attends were assessed by video by experimenters blind to treatment condition.

2.2.1.2. Forced swim test. The forced swim test was used to assess the impact of peri-adolescent AM-251 treatment on stress coping behavior. Cylindrical glass containers (diameter 35 cm × height 66 cm) were filled with water to a height of approximately 40 cm and maintained at 24 ± 1 °C. After each rat completed a session, the water was replaced. Rats were subjected to two swim sessions on PND 82 and 83. The first swim session was a 15-min pre-exposure session, followed by a 5-min test session 24 h later. During the second session, the duration of time spent engaging in passive (immobility) or active (swimming and struggling) coping behaviors (Bardi et al., 2012; Lu et al., 2008) was videotaped and scored by an experimenter blind to treatment conditions. Immobility was defined as the rat remaining stationary with minimal movement of limbs to remain afloat. Swimming was defined as paddling movement of the rat's forelimbs and/or hindlimbs in the water. Struggling was defined as quick, forceful movement of the forelimbs that broke the surface of the water.

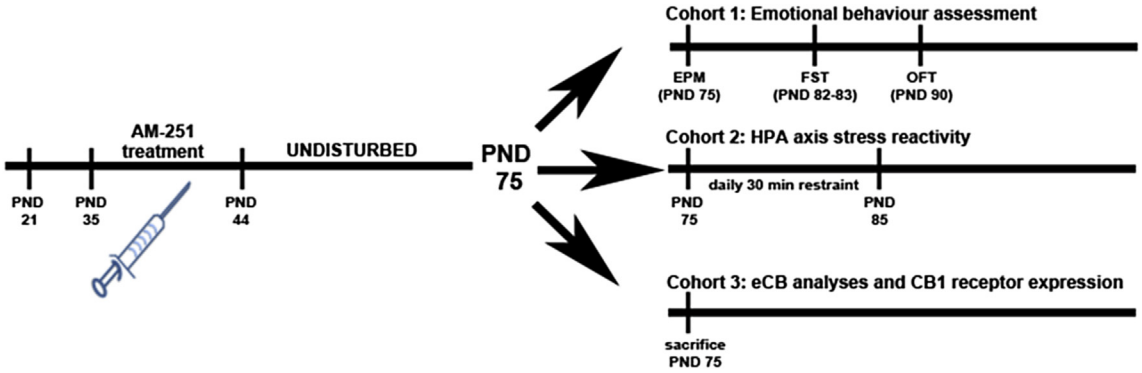

Fig. 1. Schematic diagram of the peri-adolescent treatment period and subsequent biochemical, behavioral and neuroendocrine measures. Male rats received a daily intraperitoneal injection of AM-251 (5 mg/kg) from post natal day (PND) 35–45 and were left undisturbed until adulthood. In cohort 1, rats were exposed to the elevated plus maze (EPM), forced swim test (FST) and open field test (OFT). In cohort 2, hypothalamic-pituitary-adrenal (HPA) axis stress responsivity was measured. In cohort 3, brain tissue was analyzed for endocannabinoid (eCB) content and CB_1 receptor binding.

2.2.1.3. Open field test. Rats were observed in an open field arena as an additional measure of anxiety-like behavior and general locomotor activity. The open field arena used in this study was 120 × 120 × 30 cm. The arena was painted white and divided into 16 equal quadrants (30 × 30 cm) by black lines. For testing, rats were placed in the central quadrant and left to explore for 5 min. Rats were monitored and recorded by an overhead camera (Hitachi 2500A) approximately 2 m above the open field box. A 10% acetic acid solution was used to clean the apparatus between tests. The mean number of entries and time spent in peripheral and central quadrants as well as the mean speed and total distance traveled were measured using Stoelting ANY-maze software (Wood Dale, IL).

2.2.2. HPA axis stress reactivity

All restraint stress sessions were conducted in a separate testing room, and occurred in the first third of the light cycle, during the daily nadir of HPA axis activity. During these sessions, rats were put into a polystyrene tube (diameter 6 cm, length 20 cm) with breathing holes for 10 consecutive days for 30 min each day, as described previously (Lee and Hill, 2013). On days 1 and 10 of the restraint stress paradigm, tail blood samples were taken at 0, 30, 60 and 90 min following restraint onset. Blood samples were collected in chilled EDTA- (3.75 mg/100 μL blood) and Aprotinin- (0.053 mg/100 μL blood) treated microcentrifuge tubes. All samples were centrifuged at 3000 × *g* for 15 min, after which plasma was removed and stored at −80 °C until analyses.

2.3. Corticosterone and adrenocorticotropic hormone radioimmunoassays

Total corticosterone (CORT; 5 uL) and adrenocorticotropic hormone (ACTH; 50 uL) concentrations were measured using commercial ImmuChem RIA kits (MP Biomedicals Canada), using [^{125}I] as a tracer. For CORT, plasma samples were diluted 1:100 and 1:200 for basal and stress conditions, respectively, to render hormone detection within the linear part of the standard curve (3.125–1000 ng/mL). Plasma ACTH levels were determined according to manufacturer instructions, from a standard curve derived from 8 standards ranging from 0 to 1000 pg/mL. The inter- and intra-assay coefficients of variation were all under 8% for each assay.

2.4. Endocannabinoid system content and expression

2.4.1. CB_1 receptor radioligand binding assay

Membrane preparation and CB_1R radioligand binding assays were conducted as previously described (Lee and Hill, 2013). Membranes were collected from isolated brain regions by homogenization of frozen tissue in 20 volumes of TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM $MgCl_2$) and centrifuged at 18,000 × *g* for 20 min. The resulting pellet was re-suspended in 20 volumes of TME buffer. Protein concentrations were determined using a commercially available BCA kit (Pierce Biotechnology, Rockville, IL).

CB_1R agonist binding parameters were determined by radioligand binding using a Multiscreen Filtration System with Durapore 1.2-μM filters in 96 well filter plates (Millipore, Bedford, MA). 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 in triplicate to wells containing 0.1, 0.25, 0.5, 1.0, 1.5 or 2.5 nM [^{3}H]CP 55,940 (American Radiochemicals, St. Louis, MO), a cannabinoid CB_1R agonist, and incubated for 1 h at room temperature. Ten μM AM-251 (Tocris Biosciences, Minneapolis, MN) was used to determine non-specific binding. B_{max} (maximal binding site density) and K_D (binding affinity) values were determined by nonlinear curve fitting of specific binding data to the single site binding equation using GraphPad Prism (San Diego, CA).

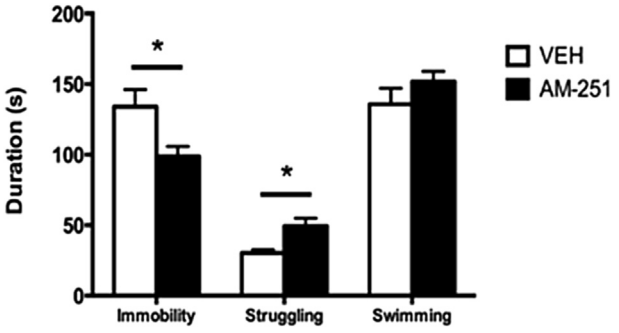

Fig. 2. Peri-adolescent AM-251 treatment increases stress-coping behavior in the forced swim test in adult male rats. Mean ± SEM time (s) spent immobile, struggling and swimming in the forced swim test. * indicates statistically significant differences at $p < 0.05$.

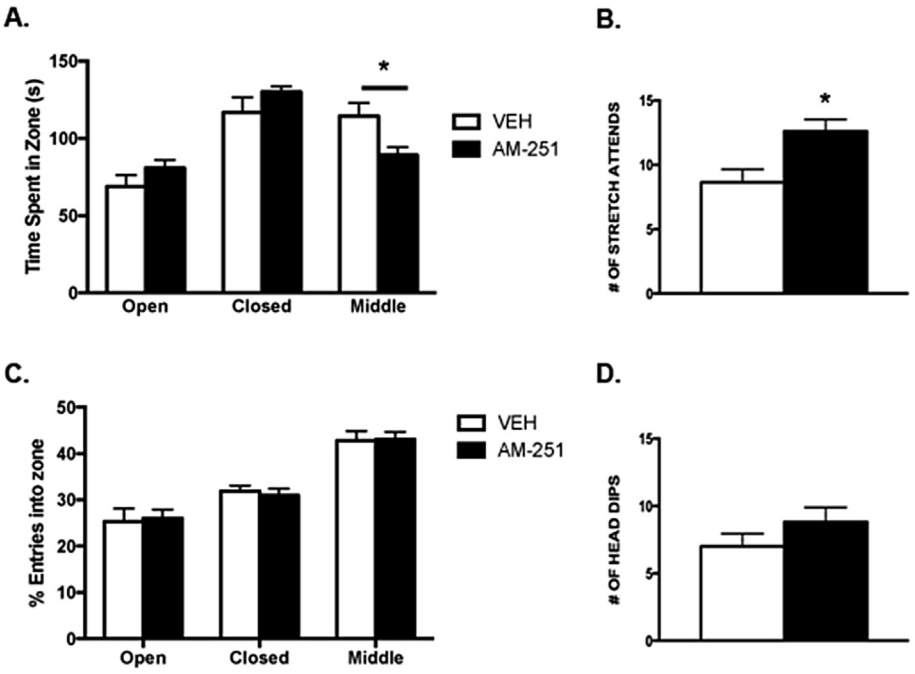

Fig. 3. Peri-adolescent AM-251 treatment increases risk assessment behavior in adult male rats. (A) Mean ± SEM time (s) spent in the open, closed and middle zones of the elevated plus maze. (B) Mean ± SEM number of stretch attends. (C) Mean ± SEM percentage of entries into the open, closed and middle zones of the elevated plus maze. (D) Mean ± SEM number of head dips. * indicates statistically significant differences at $p < 0.05$.

2.4.2. Endocannabinoid extraction and analysis

Rats were sacrificed by rapid decapitation during the first third of the light cycle and brain tissue was collected for eCB content analysis. PFC, hippocampus, amygdala, and hypothalamus were dissected within 5 min, as previously described (Hill et al., 2010; Lee et al., 2013), frozen on dry ice, and stored at −80 °C until analysis. Brain regions underwent a lipid extraction process as previously described (Patel et al., 2003). Tissue samples were weighed and placed in borosilicate glass culture tubes containing 2 mL of acetonitrile with 84 pmol of [$^2H_8$] AEA and 186 pmol of [$^2H_8$] 2-AG for extraction. These samples were homogenized with a glass rod and sonicated for 30 min, incubated overnight at −20 °C to precipitate proteins, then centrifuged at 1500 g for 5 min to remove particulates. Supernatants were removed to a new glass culture tube and evaporated to dryness under N_2 gas, re-suspended in 300 μL of methanol to recapture any lipids adhering to the tube and re-dried again under N_2 gas. The final lipid extracts were suspended in 20 μL of methanol and stored at −80 °C until analysis. AEA and 2-AG contents within lipid extracts were determined using isotope-dilution, liquid chromatography-mass spectrometry as described earlier (Patel et al., 2005).

Table 1
Mean speed and distance traveled in the open field test by adult male rats treated with AM-251 or vehicle (VEH) during adolescence.

	VEH	AM-251
Speed (m/s)	0.09 ± 0.01	0.09 ± 0.01
Distance traveled (m)	26.3 ± 3.2	28.0 ± 2.8
Time spent in center (s)	29.96 ± 6.86	29.90 ± 5.51
Time spent in peripherary (s)	270.70 ± 7.14	269.6 ± 5.56

There was no significant effect of adolescent AM-251 treatment on any of the variables measured in the open field. For all treatment conditions, n = 10. Data are presented as means ± SEM.

2.5. Data analyses

In cohort 1 and 2, all measurements were analyzed using independent t-tests. In cohort 3, HPA axis stress responsivity was analyzed using repeated measures analyses of variance (ANOVA) with blood sampling time point serving as the within-subjects variable and peri-adolescent drug treatment as the between-subjects factor. To gauge relative CORT and ACTH responses to acute and repeated restraint as a function of peri-adolescent drug treatment, total CORT and ACTH responses in the AM-251 group were determined (area under the curve) and calculated as a percentage of total CORT and ACTH response of the vehicle group for day 1 and 10 of restraint exposure. These values were analyzed using a repeated measures ANOVA with day of restraint (day 1 vs. 10) as the within subjects factor and peri-adolescent drug treatment as the between subjects factor. Bonferroni corrections were used for all post-hoc comparisons. All statistical procedures were set at $\alpha = 0.05$.

3. Results

3.1. Emotional behavior

In the forced swim test, peri-adolescent AM-251 treatment produced a significant decrease in time spent immobile ($t(19) = 2.56$, $p = 0.02$) coupled to an increase in time spent struggling ($t(19) = 3.07$, $p = 0.01$; Fig. 2) relative to vehicle-treated rats. There was no significant effect of AM-251 treatment on swimming behavior ($t(19) = 1.22$, $p = 0.24$).

Independent t-test analyses revealed no significant effect of peri-adolescent AM-251 treatment on time spent in the open ($t(19) = 1.29$, $p = 0.22$) and closed arms ($t(19) = 1.23$, $p = 0.24$) of the elevated plus maze; however, the AM-251 treated rats spent significantly less time in the middle zone than vehicle-treated rats ($t(19) = 2.50$, $p = 0.02$; Fig. 3A). There was no significant effect of

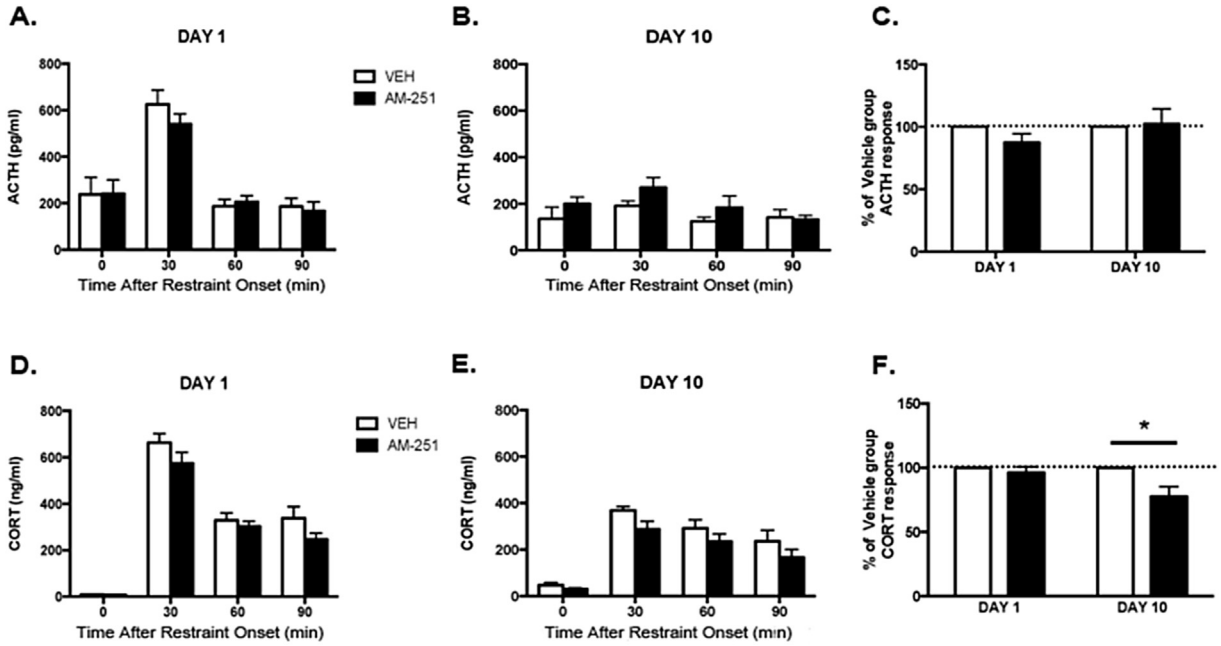

Fig. 4. Peri-adolescent AM-251 treatment modifies corticosterone (CORT) stress habituation. (A) Mean ± SEM adrenocorticotropic hormone (ACTH) levels in response to repeated restraint stress exposure on Day 1. (B) Mean ± SEM ACTH levels in response to repeated restraint stress exposure on Day 10. (C) Relative ACTH responses as percentage of the vehicle groups to acute and repeated restraint stress. (D) Mean ± SEM CORT levels in response to repeated restraint stress exposure on Day 1. (E) Mean ± SEM CORT levels in response to repeated restraint stress exposure on Day 10. (F) Relative CORT responses as percentage of the vehicle groups to acute and repeated restraint stress. * indicates statistically significant differences at $p < 0.05$.

Table 2
Mean adult CB1 receptor expression and binding affinity within corticolimbic structures of adult male rats treated with AM-251 or vehicle (VEH) during adolescence.

	B_{max} (pmol/mg protein)		K_D (nM)	
	VEH	AM-251	VEH	AM-251
Amygdala	0.68 + 0.13	0.63 + 0.06	0.54 + 0.09	0.64 + 0.07
Hippocampus	1.04 + 0.06	1.12 + 0.07	0.89 + 0.05	1.03 + 0.06
Hypothalamus	0.24 + 0.03	0.19 + 0.02	0.44 + 0.05	0.44 + 0.04
Prefrontal Cortex	0.81 + 0.05	1.00 + 0.05*	0.71 + 0.08	0.84 + 0.07

There was no significant effect of adolescent AM-251 treatment on CB1 receptor expression and binding affinity except in the prefrontal cortex. * denotes significant differences ($p \leq 0.05$). For all treatment conditions, $n = 10$. Data are presented as means ± SEM.

AM-251 treatment on the percentage of entries into the open arms ($t(19) = 0.20$, $p = 0.84$; Fig. 3C), closed arms ($t(19) = 1.88$, $p = 0.07$) and middle zone ($t(19) = 0.08$, $p = 0.94$) of the elevated plus maze. Relative to vehicle, AM-251 treatment induced significantly greater numbers of stretch attends ($t(19) = 2.89$, $p = 0.009$; Fig. 3B) but not head dips ($t(19) = 1.25$, $p = 0.23$; Fig. 3D).

No significant locomotor differences on mean speed ($t(19) = 0.39$, $p = 0.70$; Table 1) and total distance traveled ($t(19) = 0.41$, $p = 0.69$; Table 1) were detected between groups. There was also no significant effect of the AM-251 treatment on time spent in the center ($t(19) = 0.01$, $p = 0.99$; Table 1) and peripheral zones ($t(19) = 0.12$, $p = 0.91$; Table 1) of the open field.

3.2. Stress responsivity

There was a significant interaction between time point following restraint onset and peri-adolescent AM-251 treatment on adult ACTH levels ($F(9,78) = 5.33$, $p = 0.0001$; Fig. 4A, B). Post-hoc comparisons revealed no significant differences between groups at 0, 60 and 90 min following restraint onset on day 1 and 10; however, both AM-251 ($p = 0.001$) and vehicle ($p = 0.0001$) treated groups exhibited lower ACTH levels 30 min following restraint onset on day 10 relative to day 1, indicating that habituation of the HPA axis had occurred. However, there were no significant

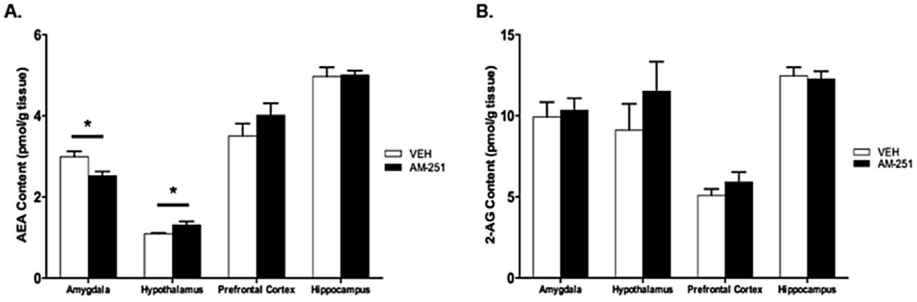

Fig. 5. Peri-adolescent AM-251 treatment alters anandamide (AEA) content in the amygdala and hypothalamus of adult male rats. (A) Mean ± SEM basal AEA and (B) 2-arachidonoylglycerol (2-AG) levels throughout adult corticolimbic brain structures following peri-adolescent AM-251 treatment. * indicates statistically significant differences at $p < 0.05$.

differences in peak stress-induced ACTH levels (30 min) between vehicle and AM-251 on day 1 ($p = 0.59$) and day 10 ($p = 0.24$) of restraint exposure. Lastly, there was no significant interaction between day of restraint and peri-adolescent treatment (area under the curve; $F(1, 12) = 1.22$, $p = 0.29$; Fig. 4C), nor any significant main effects of day ($F(1, 12) = 1.22$, $p = 0.29$) and treatment ($F(1, 12) = 1.01$, $p = 0.33$) on the magnitude of the ACTH response to restraint exposure.

There was also no significant interaction between time point following stress and peri-adolescent AM-251 treatment on adult CORT levels ($F(9,81) = 1.61$, $p = 0.13$). However, there was a significant main effect of peri-adolescent treatment ($F(3,27) = 3.62$, $p = 0.03$) such that AM-251 treated rats exhibited less of an increase in stress-induced CORT levels than the vehicle group (Fig. 4D, E). There was also a significant main effect of time after restraint, indicating habituation of stress-induced CORT increases had occurred for both groups ($F(3, 81) = 141.4$, $p < 0.0001$). There was a significant day of restraint by peri-adolescent treatment interaction on the magnitude of the CORT response ($F(1,14) = 4.71$, $p = 0.04$; Fig. 4F) such that relative to the vehicle group, the AM-251-treated rats exhibited significantly lower CORT responses to restraint on day 10 ($p = 0.005$), but not day 1 ($p > 0.05$).

3.3. Endocannabinoid system content and expression

Peri-adolescent CB_1R antagonism produced a significant increase in adult prefrontal cortical CB_1R expression (B_{max}; $t(8) = 2.91$, $p = 0.01$; see Table 2 for all regions) with no significant effects in the amygdala ($t(7) = 0.51$, $p = 0.63$), hypothalamus ($t(8) = 1.06$, $p = 0.31$) and hippocampus ($t(8) = 0.82$, $p = 0.44$). Peri-adolescent AM-251 treatment did not significantly affect binding affinity (K_D) within the amygdala ($t(8) = 1.19$, $p = 0.27$; see Table 2 for all regions), hippocampus ($t(8) = 1.78$, $p = 0.11$), hypothalamus ($t(8) = 0.09$, $p = 0.93$), or PFC ($t(8) = 1.19$, $p = 0.27$).

Independent t-test analyses revealed a significant increase in adult hypothalamic AEA levels ($t(8) = 2.51$, $p = 0.03$; see Fig. 5 for all regions) and significant decrease in amygdalar AEA ($t(8) = 2.72$, $p = 0.03$). However, there was no effect of AM-251 treatment on adult AEA levels in the PFC ($t(8) = 1.12$, $p = 0.30$) nor the hippocampus ($t(8) = 0.14$, $p = 0.89$). Peri-adolescent AM-251 treatment had no significant effect on adult 2-AG levels in the amygdala ($t(8) = 0.35$, $p = 0.74$; see Fig. 5B for all regions), hypothalamus ($t(8) = 0.97$, $p = 0.36$), PFC ($t(8) = 1.17$, $p = 0.30$) and hippocampus ($t(8) = 0.28$, $p = 0.79$).

4. Discussion

Results of the current study indicate that disruption of peri-adolescent eCB signaling has moderate organizational effects in altering the developmental trajectory of the adult corticolimbic eCB system, emotional behavior, and stress responsivity. Sustained CB_1R blockade during peri-adolescence increased active stress-coping behavior in the forced swim test, while moderately increasing risk assessment behavior in the elevated plus maze, but not the open field test, in adulthood. Additionally, the peri-adolescent AM-251 treatment had no effect on the acute HPA axis stress response, but did modify habituation of CORT secretion in response to repeated restraint stress. Lastly, disruption of peri-adolescent eCB signaling decreased amygdalar AEA, increased hypothalamic AEA content, and increased CB_1R expression in the PFC of adult rats.

Sustained CB_1R blockade during adolescence increased active stress coping behavior in the forced swim test in adulthood. These findings are consistent with previous work demonstrating that adolescent CB_1R agonist treatment decreases active coping behavior in adult female rats (Rubino et al., 2008), suggesting that pharmacological modulation of eCB signaling during adolescence affects stress coping behaviors in adulthood. Given that CB_1R signaling in the PFC has been shown to increase active stress coping in the forced swim test (Bambico et al., 2007; McLaughlin et al., 2012), it is possible the observed increase in PFC CB_1R expression could result in this behavioral alteration. Furthermore, the increase in active coping behavior was specifically associated with an increase in struggling behavior, suggesting a noradrenergic mechanism, given the importance of norepinephrine signaling in regulating struggling behavior (Detke et al., 1995). Interestingly, CB_1R is co-localized with noradrenergic terminals in the PFC where CB_1R activation enhances norepinephrine release (Oropeza et al., 2007; Page et al., 2008). Given this relationship, the possibility exists that disruption of adolescent eCB signaling modulates the interaction between frontocortical eCB and noradrenergic systems to enhance active coping responses to stress in adulthood.

Unlike the effects on stress coping behavior, disruption of peri-adolescent eCB signaling did not alter gross measures of anxiety-like behavior (i.e., entries or time spent in the open and closed arms). AM-251-treated rats, however, did exhibit greater risk assessment behavior as revealed by an increase in stretch attends to the open arms, but less time spent in the middle zone relative to those in the vehicle group. Interestingly, stretch attends and head dips are largely observed when the rat is in the middle zone of the elevated plus maze (Blanchard et al., 2003; Rodgers and Dalvi, 1997), suggesting that sustained adolescent CB_1R blockade has the capacity to alter normative rodent risk assessment strategies in the elevated plus maze and their frequency. Nonetheless, these findings are generally consistent with previously reported effects of adult AM-251 treatment on anxiety-like behavior. However, in contrast to the present findings, chronic adult AM-251 treatment has been previously reported to produce a negative correlation between PFC CB_1R expression and time spent in the center of the elevated plus maze and stretch attend postures (Tambaro et al., 2013). Despite this, increased risk assessment following disruption of peri-adolescent eCB signaling in the current study is reminiscent of the effects of stress during adolescence, which has been shown to increase risk assessment behavior and corticolimbic noradrenergic activity (Bingham et al., 2011; Watt et al., 2009). One interpretation of these data is that disruption of adolescent eCB signaling could generate a state of stress that produces similar effects on the adult brain as exposure to stress itself during adolescence. Given that stress exposure during peri-adolescent windows has been shown to transiently impair the eCB system (Lee and Hill, 2013; Wamsteeker et al., 2010) and the parallels between the effects of stress and disruption of eCB signaling during adolescence, it is possible that stress-induced impairments in eCB signaling during adolescence contribute to the sustained effects of stress on the adult brain.

With respect to adult regulation of anxiety, there is evidence that AEA signaling can produce reductions on indices of stress and anxiety through signaling actions within the amygdala (Bedse et al., 2014; Hill et al., 2009). Given that the peri-adolescent treatment did not dramatically modulate anxiety-like behavior, it is possible this modest reduction in amygdalar AEA is contributing to moderately higher levels of anxiety-like behavior observed in the AM-251 group. Furthermore, given that the AM-251 group exhibited an increase in PFC CB_1R expression and lower amygdalar AEA content, it is possible peri-adolescent CB_1R blockade induced dysregulation between PFC-amygdala connectivity that elicited greater risk-assessment behavior and a tendency towards more time spent in the closed arms of the elevated plus maze. However, further research assessing this possibility is required.

Analyses of basal and acute stress-induced increases in ACTH and CORT levels did not yield any significant treatment differences between AM-251 and vehicle. However, AM-251–treated rats exhibited a lower CORT response with repeated restraint exposure, despite no treatment differences on the first day of restraint exposure. These findings indicate that peri-adolescent CB_1R antagonism modified HPA axis stress habituation. Interestingly, the modified recovery from repeated restraint stress is somewhat reminiscent of the immature phenotype (Spear and Swartzwelder, 2014) in which prepubertal rats exhibit an accelerated return to non-stress levels when exposed to repeated restraint relative to adults (Romeo et al., 2006); however, the expression of HPA axis habituation remained intact in the adult AM-251 treated rats (in the current study), unlike in prepubertal rats. Furthermore, while corticolimbic eCB content was not measured during exposure to restraint stress, the increase in basal hypothalamic AEA following adolescent AM-251 treatment may promote greater negative feedback on HPA axis habituation, thus resulting in a lower CORT response to repeated restraint stress. This hypothesis is consistent with the role of CB_1R signaling in the PVN to facilitate negative feedback of the HPA axis (Di et al., 2003; Evanson et al., 2010). Similarly, PFC CB_1R signaling has been shown to be important for feedback inhibition of stress-induced CORT secretion (Hill et al., 2011b), thus the increase in PFC CB_1R binding following peri-adolescent AM-251 treatment may also be contributing to this effect.

Chronic administration of a CB_1R agonist during adolescence downregulates multiple components of the adult eCB system (Rubino et al., 2008). Our data indicate peri-adolescent blockade of CB_1Rs also results in alterations in the adult eCB system, suggesting that the establishment of steady-state eCB signaling in adulthood is sensitive to alterations in adolescent eCB signaling. These data parallel similar studies that have examined the effects of sustained CB_1R blockade in adulthood. Chronic treatment with AM-251 in adult mice induced region-dependent differences in CB_1R expression, with an upregulation in the PFC and striatum and down-regulation in the hippocampus and mid-brain (Tambaro et al., 2013). Together, the findings suggest that sustained CB_1R blockade in both adolescence and adulthood elicit an upregulation of prefrontal cortical CB_1R expression.

Lastly, the current study revealed that peri-adolescent AM-251 treatment significantly reduced and increased AEA in the adult amygdala and hypothalamus, respectively. In contrast, chronic low dose AM-251 treatment from late-adolescence to young adulthood (PND 56–77) failed to alter AEA and 2-AG content in corticolimbic structures of group housed, but not isolation-reared adult male rats (Zamberletti et al., 2012a). This would suggest that there is differential sensitivity in the age through which alterations in adolescent eCB signaling alter expression of the adult eCB system. Importantly, the current results also suggest peri-adolescent (PND 35-45) AM-251 treatment induced long lasting alterations in adult eCB signaling within the amygdala and PFC, which are key structures regulating emotionality and stress responsivity and are among the last neural structures to reach adult structural and functional maturity (Gogtay et al., 2004).

The present findings suggest that adolescent CB_1R activation plays a moderate, yet significant organizational role in the normative development of the corticolimbic endocannabinoid system, emotionality and stress responsivity. Results of the current study and previous work indicate adults may be more susceptible to the deleterious effects of sustained CB_1R blockade given that the neural and behavioral alterations reported in the current study are relatively modest, and are generally associated with what are typically viewed as more adaptive responses to stress (i.e., increased active stress coping behaviors). Furthermore, while future research examining the mechanisms of action is necessary, it is possible that there are some adaptive aspects of the adolescent AM-251 treatment. For example, there is some evidence that chronic AM-251 treatment reverses isolation rearing-induced deficits in cognition and sensorimotor gating and normalizes eCB, dopaminergic and glutamatergic imbalances associated with this model of psychotic-like symptoms in male and female rats (Guidali et al., 2011; Zamberletti et al., 2012a, 2012b).

Acknowledgments

The authors wish to thank Tori Wong for assistance with this work. The authors would also like to thank the following sources of funding support: TTYL – Canadian Institute of Health Research (CIHR) and the Canadian Consortium for the Investigation of Cannabinoids (CCIC; salary); MNH – Natural Sciences and Engineering Research Council (NSERC) and CIHR (operating); CJH – the Research Component of the Advancing a Healthier Wisconsin endowment at the Medical College of Wisconsin; BBG – NSERC and CIHR (operating; MOP 79399).

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