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Effects of an early life experience on rat brain cannabinoid receptors in adolescence and adulthood

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ABSTRACT

Neonatal handling is an experimental model of early life experience associated with resilience in later life challenges, altering the ability of animals to respond to stress. The endocannabinoid system of the brain modulates the neuroendocrine and behavioral effects of stress, while this system is also capable of being modulated by stress exposure itself. The present study has addressed the question of whether neonatal handling in rats could affect cannabinoid receptors, in an age- and sex-dependent manner, using *in situ* hybridization and receptor binding techniques. Different effects of neonatal handling were observed in adolescent and adult brain on CB1 receptor mRNA and [^{3}H]CP55,940 binding levels, which in some cases were sexually dimorphic. Neonatal handling interfered in the developmental trajectories of CB1 receptor mRNA levels in striatum and amygdaloid nuclei, as well as of [^{3}H]CP55,940 binding levels in almost all regions studied. Adult handled rats showed reduced [^{3}H]CP55,940 binding levels in the prefrontal cortex, striatum, nucleus accumbens and basolateral amygdala, while binding levels in prefrontal cortex of adolescent handled rats were increased. Finally, handling resulted in decreases in female [^{3}H]CP55,940 binding levels in the striatum, nucleus accumbens, CA3 and DG of dorsal hippocampus and basolateral amygdala. Our results suggest that a brief and repeated maternal separation during the neonatal period induces changes on cannabinoid receptors differently manifested between adolescence and adulthood, male and female brain, which could be correlated to their stress response.

1. Introduction

Neonatal handling is an experimental model of early life experience, originally developed by Levine, in which pups are separated from their mother briefly (15 min daily) during the first 21 days of life (Levine, 1957). This brief separation is considered as an early life experience associated with resilience in later life challenges, altering the hypothalamic-pituitary-adrenal axis function and the ability of animals to respond to stress. Specifically, neonatally handled adult animals have increased numbers of glucocorticoid receptors (GR) in the brain (Meaney and Aitken, 1985; Meaney et al., 1985b; Wilber et al., 2008) and consequently they tend to secrete less corticotropin-releasing hormone, adrenocorticotropic hormone and corticosterone following exposure to stressful stimuli, due to enhanced sensitivity of the negative feedback loop (Plotsky and Meaney, 1993; Bhatnagar and Meaney, 1995; Vallée et al., 1996, 1997; Liu et al., 1997). In addition, neonatally handled animals show increased explorative behavior, decreased fear and/or anxiety and an enhanced ability to cope with stressful events (Chapillon et al., 2002; Meaney et al., 1991; Fernandez-Teruel et al., 1997; Vallée et al., 1997; Meerlo et al., 1999). Several studies have shown that neonatal handling enhances spatial learning and memory (Escorihuela et al., 1995; Pryce et al., 2003; Beane et al., 2002; Wong and Jamieson, 1968; Huot et al., 2002; Fenoglio et al., 2005). There is strong evidence that neonatal handling has a number of sexually

Abbreviations: 2-AG, 2-arachidonoylglycerol; ANOVA, analysis of variance; BLA, basolateral nucleus of amygdala; BSA, bovine serum albumin; CA1, dorsal field 1 of Ammon's horn; CA3, dorsal field 3 of Ammon's horn; CB1, cannabinoid receptor 1; CeA, central amygdaloid nucleus; Cg1, anterior cingulate cortex; CPu-DL, dorsolateral striatum; CPu-VM, ventromedial striatum; DG, dentate gyrus; eCB, endocannabinoid; GR, glucocorticoid receptors; GrDG, dentate gyrus granule cell layer; HPA, hypothalamic-pituitary-adrenal; IL, infralimbic cortex; LTD, long-term depression; mPFC, medial prefrontal cortex; MO, medial orbital cortex; MoDG, dentate gyrus molecular layer; NAc, nucleus accumbens; NS, not significant; PFC, prefrontal cortex; PND, postnatal day; PrL, prelimbic cortex; ROD, relative optical density; RT, room temperature

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dimorphic effects, such as on the serotoninergic system (Smythe et al., 1994; Stamatakis et al., 2006), stress reactivity (Papaioannou et al., 2002; Park et al., 2003,) learning and memory (Kosten et al., 2007; Stamatakis et al., 2008) and extinction of fear (Stevenson et al., 2009).

The endocannabinoid system is a neuromodulatory system that consists of cannabinoid receptors [mainly cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2)], endogenous cannabinoid ligands, among which the best characterized are anandamide and 2-arachidonoylglycerol (2-AG), a putative membrane transporter, and enzymes involved in the synthesis and inactivation of the endogenous ligands (Di Marzo, 2006; Hillard, 2015; Piomelli, 2003). The endocannabinoid system of the brain represents an important regulator of the adult hypothalamic-pituitary-adrenal (HPA) axis stress response, modulating the neuroendocrine and behavioral effects of stress, while this system is also capable of being modulated by stress exposure itself (Hill et al., 2010a,b; Hill and Tasker, 2012; Riebe and Wotjak, 2011). Furthermore, it is an important substrate for the control of emotional behavior and mood (Marsicano et al., 2002; Valverde and Torrens, 2012), it is involved in brain reward processes and drug addiction (Solinas et al., 2007, 2008) and it plays a specific role in neural development, guiding the establishment of cortical-subcortical connections (Belue et al., 1995; Mato et al., 2003; Rodríguez de Fonseca et al., 1993). There is evidence that early life experiences of maternal deprivation and social isolation have an effect on several parameters of the endocannabinoid system of the neonatal (Suárez et al., 2009), adolescent (Marco et al., 2014) and adult rat brain (Robinson et al., 2010), while the effect of maternal deprivation is gender dependent.

Given that the endocannabinoid system is important in the regulation of stress and is also being modulated by stress exposure, the present study addressed the question of whether neonatal handling in rats, which is known to confer resilience to stress, could affect in the long-term the endocannabinoid system. In particular, we were interested to investigate whether handling-induced changes in cannabinoid receptors (CB1 mRNA expression and [^{3}H]CP55,940 binding levels) are differently manifested between adolescence and adulthood, as well as between male and female brain. Our findings indicate that the consequences of neonatal handling on rat brain CB1 receptors are age and sex-dependent.

2. Materials and methods

2.1. Animals

Wistar rats of both sexes were reared in the Animal Facility of the Medical School of the University of Patras (Patras, Greece) under standard conditions (21 ± 1 °C; 12 h light/dark cycle, lights on at 08:00 h) and received food and water *ad libitum*. Two or three virgin females were housed with one stud male rat. Pregnant females were caged separately and litters were randomly distributed to either the handled or non-handled groups. A total of 9 litters were used for the experiment (4–5 litters in each of the two groups: handled, non-handled). The litter size and the sex ratio did not significantly differ between the litters employed in the two groups [average litter size [mean ± SEM (standard error of the mean): non-handled litters 11.75 ± 0.19 (range, 11–12), handled litters 10.6 ± 0.32 (range, 9–12); average sex ratio (males:females, mean ± SEM): non-handled litters 2.15 ± 0.64; handled litters 1.06 ± 0.10]. Culling of litters was not performed since it has been shown that litter size within the range employed does not affect maternal behavior (Champagne et al., 2003; Deviterne et al., 1990). The day of birth was determined as postnatal day 0. Following weaning, three to four animals of the same sex, litter, and group (handled or non-handled) were placed per cage and were kept under standard housing conditions in the same room. A total of 56 animals were used in the present study: 28 mid-adolescent (PND 39–40) and 28 adult animals (PND 89–90). For each age group seven male non-handled, seven male handled, seven female non-handled, and seven female handled rats were used. In each group, animals from all litters were employed. Experiments were carried out in agreement with the ethical recommendation of the European Communities Council Directives of November 24, 1986 (86/609/EEC) and of September 22, 2010 (2010/63/EU). All efforts were made to minimize the number of animals used and their suffering.

2.2. Neonatal handling

We employed a neonatal handling protocol similar to that originally described by Levine (1957) lasting from PND1 until weaning (PND21) and recently described by Katsouli et al. (2014). In particular, every day between 9:00–10:00 a.m. the mothers of the pups were removed from their home cages and placed separately into cages left in the same room (always the same cage for each mother throughout the handling period). All offsprings of a litter were then removed, placed together in a clean plastic container and heated by a lamp so that the temperature close to the pups was 28–29 °C. After 15 min, the pups and then their mothers were returned to their home cages. Non-handled litters were left completely undisturbed until weaning.

2.3. Tissue preparation

On PND39-PND40 or on PND89-PND90 rats were deeply anesthetized with isofluorane, decapitated and the brains were isolated and flash-frozen in −50 °C isopentane. Brain tissue was kept at −80 °C, until use. Brain tissue was cut into coronal 15 μm sections on a cryostat (Leica CM1500, Germany) at −18 °C, thaw mounted onto 0.01% poly-L-lysine-coated slides (for *in situ* hybridization experiments) or on acid-clean gelatin-coated slides (for *in vitro* receptor binding experiments). Sections were allowed to air-dry at room temperature and stored at −80 °C until further processing.

Four coronal sections were collected non-consecutively on each slide, so both rostral and caudal parts of each brain region were represented. Sections were collected separately at three different brain levels based on a brain atlas (Paxinos and Watson, 2007) which included the prefrontal cortex (AP 4.68–AP 3), the striatum (AP 2.04 to AP −0.24) and the hippocampus (−2.52 to AP −3.36).

2.4. In vitro receptor binding

In vitro receptor binding for cannabinoid receptors was performed as previously described (Dalton and Zavitsanou, 2010). Cannabinoid receptor binding levels were evaluated using quantitative *in vitro* receptor autoradiography and the radioligand [^{3}H]CP55,940 (specific activity 141.2 Ci/mg, PerkinElmer, USA), a potent, non-selective agonist, which activates both CB1 and CB2 receptors with equal potency (Howlett et al., 2002).

Sections were preincubated in 50 mM Tris-HCl containing 5% BSA, pH7.4 at RT; air-dried and incubated in the same buffer containing [^{3}H]CP55,940 at final concentration 7.37 nM (prefrontal cortex), 7.24 nM (striatum), 6.75 nM (hippocampus). *Non*-specific binding was determined by incubating adjacent sections to the buffer containing the tritiated ligand plus 10 μM CP55,940. Three post-incubation washes at 4 °C were performed as follows: 1 h in 50 mM Tris HCl (pH 7.4) plus 1% BSA; 3 h in 50 mM Tris HCl (pH 7.4) plus 1% BSA; 5 min in 50 mM Tris HCl (pH 7.4). In each assay brain sections from all four categories of either male or female animals (handled, non-handled and adult, adolescent) were processed concurrently.

2.5. In situ hybridization

The oligodeoxyribonucleotide probe used in the present study was 48 base-long and was complementary to the mRNA encoding the rat CB1 subtype of cannabinoid receptors. The sequence of the synthetic oligonucleotide was: