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# PARTICIPATION OF ENDOCANNABINOIDS IN RAPID SUPPRESSION OF STRESS RESPONSES BY GLUCOCORTICOIDS IN NEONATES

**A. BUWEMBO,[b] H. LONG[a] AND C.-D. WALKER[a]***

[a] *Douglas Mental Health University Institute, Department of Psychiatry, McGill University, Montreal, Canada*

[b] *Integrated Program in Neuroscience, McGill University, Montreal, Canada*

**Abstract—In adult rodents, endocannabinoids (eCBs) regulate fast glucocorticoid (GC) feedback in the hypothalamus–pituitary adrenal (HPA) axis, acting as retrograde messengers that bind to cannabinoid receptors (CB1R) and inhibit glutamate release from presynaptic CRH neurons in the paraventricular nucleus of the hypothalamus (PVN). During the first two weeks of life, rat pups exhibit significant CRH and ACTH responses to stress although the adrenal GC output remains reduced. At the same time, pups also display increased sensitivity to GC feedback, but it is unclear whether eCBs play a role in mediating fast GC feedback in neonatal life. In our studies, we examined the role of eCBs in the rapid suppression of anoxia-induced ACTH release and determined whether eCB action could be modulated by the levels of circulating GCs present at the time of stress. PND8 pups were subjected to 3-min anoxia with AM251, a CB1R blocker, injected 30 min prior to stress onset. The effects of either metyrapone (MET) (a steroidogenic 11beta-hydroxylase blocker) or methylprednisolone (PRED) (a synthetic GC) pretreatment on AM251 effect and the stress response were evaluated. Treatment with AM251 before stress onset tended to increase overall ACTH and CORT secretion, and also delayed the return to baseline ACTH. The AM251 effect on ACTH in PND8 pups was lost in MET-treated pups, who exhibited high basal and stimulated ACTH release and no CORT response to stress. Methylprednisolone suppressed ACTH stress responses although AM251 still delayed restoration of ACTH levels to the baseline. This suggests that the eCB effect on ACTH secretion in neonates is most evident when there is a dynamic fluctuation of corticosterone levels. Interestingly, AM251 increased basal and stimulated corticosterone secretion in all treatments including MET, suggestive of a direct action of CB1R blockade on adrenal steroidogenesis.**

*Corresponding author. Address: Douglas Mental Health University Institute, 6875 Lasalle Boulevard Montreal, QC, Canada H4H 1R3.
E-mail addresses: Dominique.walker@douglas.mcgill.ca, waldom@douglas.mcgill.ca (C.-D. Walker).

*Abbreviations:* 2-AG, 2-arachidonoylglycerol; ACTH, adrenocorticotropic hormone; AEA, *N*-arachidonylethanolamine anandamide; ANOVA, analysis of variance; CB1R, cannabinoid type 1 receptor; CORT, corticosterone; CRH, corticotropin-releasing hormone; DMSO, dimethyl sulfoxide; DOC, deoxycorticosterone; eCB, endocannabinoid; GC, glucocorticoid; GR, glucocorticoid receptor; HPA, hypothalamus–pituitary adrenal; MET, metyrapone; MR, mineralocorticoid receptor; PND, postnatal day; PRED, methylprednisolone; PVN, paraventricular nucleus of the hypothalamus; veh, vehicle.

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**Key words: stress, endocannabinoids, neonatal rat, glucocorticoids, metyrapone.**

## INTRODUCTION

Stress responses constitute integral and essential physiological reactions to conditions that threaten homeostasis and survival. Stress-activated limbic and subcortical brain regions send converging inputs on CRH (corticotropin-releasing hormone)-secreting neurons of the paraventricular nucleus of the hypothalamus (PVN) (Herman et al., 2003), which stimulate pituitary adrenocorticotropic hormone (ACTH) and adrenal corticosterone (CORT) secretion (Ulrich-Lai and Herman, 2009). Glucocorticoids provide an inhibitory feedback signal on the hypothalamus–pituitary adrenal (HPA) axis at the central and peripheral sites and within various time frames including fast (seconds to minutes), intermediate (minutes to hours) and delayed (hours to days) feedback domains (Keller-Wood and Dallman, 1984; Grino et al., 1989; De Kloet et al., 1998; Tasker et al., 2006; Groeneweg et al., 2011). While cytoplasmic glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) are known to mediate the classical genomic actions of glucocorticoids (GCs) within the intermediate and delayed feedback domains (Reul and de Kloet, 1985; Groeneweg et al., 2011), rapid actions of GCs within the fast feedback domain are thought to be mediated by membrane GRs with actions on cellular excitability (Hinz and Hirschelmann, 2000; de Kloet et al., 2008) and the production of retrograde signaling molecules such as endocannabinoids (eCBs) (Di et al., 2003; Malcher-Lopes et al., 2006). Indeed, GR agonists were shown to suppress ACTH secretion *in vivo* within minutes of peripheral injection (Ginsberg et al., 2010) and intracerebroventricular injections of dexamethasone:BSA rapidly suppressed stress-induced ACTH secretion only when eCB action was allowed (Evanson et al., 2010).

eCBs are lipid molecules derived from arachidonic acid that are synthesized upon cellular excitation and affect presynaptic neurotransmitter release (Hillard, 2000). The distribution of the two main eCBs *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) as well as their

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degradation enzymes show regional specificity within the brain (Hillard, 2000; Ueda, 2002). eCBs bind specific presynaptic CB1 receptors (CB1R) in several brain regions (Moldrich and Wenger, 2000; Di et al., 2003; Patel et al., 2004), and they mostly reduce the firing rate of glutamatergic and GABAergic terminals through inhibition of voltage-gated calcium channels (Freund et al., 2003). In adult rats, stress-induced changes in eCB concentrations have been documented in the amygdala, hippocampus, prefrontal cortex and hypothalamus, all of which play a functional role in regulation of the HPA axis (Evanson et al., 2010; Hill et al., 2010). However, changes in AEA and 2-AG are not unidirectional in all structures (Patel et al., 2005; Hill et al., 2009; Wang et al., 2012). While reductions in AEA in the basolateral amygdala are thought to mediate HPA axis activation (Hill et al., 2009), stress-induced increases in hypothalamic 2-AG concentrations are thought to be critical in mediating fast-feedback inhibition of the HPA axis (Malcher-Lopes et al., 2006; Hill et al., 2010).

eCBs are critically involved in many developmental processes. Pre- or perinatal manipulations of eCBs and the CB1R in the developing brain have effects that may be detrimental to growth (Fride, 2008), behavioral regulation (McGregor et al., 1996; Kathuria et al., 2003; Trezza and Vanderschuren, 2008; Lee and Gorzalka, 2012) and even survival (Bernard et al., 2005) of the offspring. In a previous report, we demonstrated that the nature of maternal dietary fat during the perinatal period could influence the concentration of 2-AG and AEA in the neonatal hypothalamus and hippocampus and that treatment with a CB1R antagonist (AM251) increased the baseline secretion of ACTH in postnatal day (PND) 10 offspring similarly to the adult (D'Asti et al., 2010). However, in this previous study, we did not examine the relationship between eCBs and the GC milieu at the time of stress and the potential effect of CB1R antagonist on stress-induced ACTH secretion within a fast GC feedback range. This is of particular importance since neonates exhibit a reduced GC response to stress during the first two weeks of life (Schoenfeld et al., 1980; Walker et al., 1986a, 1991) and their sensitivity to GC feedback in the intermediate range is enhanced (Walker et al., 1986b). Following the adrenal stress hyporesponsive period, the magnitude of both ACTH and CORT secretion post-stress increases through adolescence and into adulthood (Walker et al., 1986a; Vazquez and Akil, 1993). Interestingly, the hormonal response to stress during adolescence is characterized by delayed recovery to baseline compared to that observed in adult rodents suggestive of impaired GC feedback (Vazquez and Akil, 1993; Romeo et al., 2004a,b; Foilb et al., 2011) and a possible reduction in the eCB tone at that age (Wenger et al., 2002; Ellgren et al., 2008; Lee et al., 2012). Thus, examining the pre-weaning regulation of the HPA axis by eCBs provides a better understanding of the potential mechanisms allowing for specific changes in eCBs that are observed in the peri-adolescence stage. Furthermore, while earlier studies have demonstrated highly efficient perinatal GC feedback in the intermediate range, very few studies have investigated a shorter time frame of GC action in neonates.

The goal of the present experiments was to determine whether eCBs participate in the fast-feedback effect of GCs on the stress response and whether their action can be modulated by the levels of circulating GCs present at the time of stress. Thus, we tested the effect of AM251, a CB1R antagonist, on ACTH and CORT responses of PND8 neonates to anoxia stress under conditions of pharmacological adrenalectomy or conditions of high-circulating GC concentrations following injection with methylprednisolone (PRED). A second objective of this research was to examine whether the timing of AM251 injection prior to stress modified the effect of this antagonist on neonatal ACTH stress responses since in adult rats, only a short (2 min), but not a long (60 min) interval, between AM251 and restraint stress increased ACTH stress responses (Ginsberg et al., 2010).

## EXPERIMENTAL PROCEDURES

### Animals

Pregnant Sprague–Dawley rats (Charles River, St. Constant, Quebec, Canada) were received on gestation day 17–18 and maintained under controlled conditions of light (08:00 lights on, 20:00 lights off), temperature (22–24 °C) and humidity (70–80%). Mothers were fed an *ad libitum* control (60% carbohydrates, 5% fat, 15% protein) powdered diet (Harlan Teklad, Madison, WI, USA) throughout the remainder of gestation and lactation. Once the pups were born, litters were culled to 10–11 pups per mother on PND 1. Experimental procedures were conducted in the morning on male and female pups aged PND7-8. All procedures were approved by the University Animal Care Committee at the McGill University and followed ethical guidelines from the Canadian Council on Animal Care.

### Drugs

Drug treatments were given intraperitoneally (i.p.) in a volume of 50 µl 30 min prior to stress onset except for metyrapone (MET) which was injected at −12 and −1 h prior to stress The specific CB1R antagonist used was AM251 (3 mg/kg in DMSO:saline 1:2.5, for every 1 part DMSO there was 2.5 parts saline 0.9%, Tocris Biosciences). Methylprednisolone sodium succinate (PRED, 15 mg/kg in saline 0.9%, Sigma) was used to increase GR occupancy and Metyrapone (MET, 100 mg/kg in saline 0.9%, Tocris Biosciences) was used to block the endogenous production of CORT. Control groups were injected with either vehicle (DMSO:saline) or saline 0.9%.

### Stress procedure

Pups from several litters were separated from their mothers and brought to the procedure room in a large cage maintained on a warming pad. Experimental pups (including males and females) were not separated from their mothers for more than 1hr until injected or exposed to stress. Stress consisted of a 3-min exposure to a flow of N2 in a closed container kept in a water bath maintained at 30–32 °C. Pups were decapitated either without exposure to stress or at various times after stress onset (5, 15, and 30 min) for trunk blood collection. Blood was collected into Eppendorff tubes containing 10 µl of EDTA (60 mg/ml). Plasma was separated and kept at −20 °C for subsequent determinations of ACTH and CORT concentrations.